NUCLEAR MAGNETIC RESONANCE STUDIES OF NATIVE AND DENATURED PROTEINS

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I certify that this thesis is my own original work, except where due reference is made to the work of others.

N L King

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Further specific acknowledgements are included in the text.

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SUMMARY

By means of NMR difference spectroscopy, the C-4 imidazole peaks of ribonuclease are separated from overlapping aromatic resonances. The C-4 peaks provide information complementary to that gained from the C-2 peaks by previous workers (e.g. Bradbury & Scheraga, 1966; Meadows et al., 1969; Ruterjans & Witzel, 1969). The dipolar contribution to peak widths in protein spectra is discussed, with particular reference to the influence of protons close to imidazole rings in native proteins.

An NMR method for detecting residual non-covalent interactions between amino-acid side-chains is proposed, and the method is applied to a range of proteins unfolded by a number of denaturants. For several types of conformational changes, the significance of NMR transition curves is examined. Intermediate stages in the unfolding of proteins can be recognised by criteria peculiar to NMR spectra. The unfolding of ribonuclease by urea and GuCl in the pH region 4-5 at 33.4°C is found to be single-step, but with potassium thiocyanate and formic acid as denaturants, intermediate stages are

present. Likewise, intermediates are involved in the unfolding of lysozyme and α-lactalbumin B by urea. To a certain extent, NMR spectra support the model proposed for α-lactalbumin B by Browne et al. (1969).

ABBREVIATIONS

d-	Deutero-
DCA	Dichloroacetic Acid
FA	Formic Acid
GuCl	Guanidine Hydrochloride
NMR	Nuclear Magnetic Resonance
RNASE	Ribonuclease
S	Spinning Side-band
TFA	Trifluoroacetic Acid

General Introduction

The determination of molecular structure is a problem basic to many other studies in protein chemistry, e.g. enzyme mechanisms, antigen-antibody complexes, and drug-receptor interactions. Considerable advances in this field have been made in recent times, and the spatial configuration of atoms in several crystalline proteins is now known to a reasonable degree of accuracy (Venkatachalam & Ramachandran, 1969). Once established, these configurations represent definitive results, at least for proteins in the solid state.

In vivo, however, most proteins exist in solution, and there they may undergo conformational changes under the influence of a variety of agents (e.g. substrates and denaturants). While X-ray diffraction remains an invaluable technique for the determination of molecular structure in the solid state, methods for studying conformation in solution have lagged far behind.

One method being developed in an attempt to overcome this lag is that of nuclear magnetic resonance (NMR). Its application to proteins and other macromolecules has recently been reviewed by Markley et al. (1969) and

Sheard & Bradbury. The value of NMR stems from the fact that several amino acids give rise to characteristic peaks in the spectrum. Alterations in the relative position or shape of a peak reflect chemical or physical changes affecting the corresponding amino-acid residue in the protein. Depending on the number and distribution of each type of side-chain in the three-dimensional structure, NMR spectra can sometimes be used to distinguish between different regions of a protein molecule.

Other physicochemical methods are less discriminating. They yield information either about the molecule as a whole (e.g. viscometry, ultracentrifugation and optical rotatory dispersion) or about aromatic groups only (ultraviolet and fluorescence spectroscopy).

The chief disadvantage of NMR is that protein solutions of high concentration (10%) are required, and some proteins form aggregates or gels in such solutions, while others are insufficiently soluble. Another disadvantage is that most peaks are composite ones, consisting of broad, overlapping resonances. These difficulties are being overcome by the development of spectrometers with higher sensitivity and stronger magnetic fields (Ferguson & Phillips, 1967).

The general aim of this thesis is to apply the technique of nuclear magnetic resonance to susceptible problems in protein structure.

CHAPTER 1

THEORY

1A NMR

A comprehensive account of the theory of nuclear magnetic resonance is given by Abragam (1961) while numerous texts describe its applications in chemistry (e.g. Pople et al., 1959; Emsley et al., 1965; Bovey, 1969).

Factors determining polymer peak widths are summarised in this section.

The width of a peak in a protein spectrum may be regarded as the sum of a number of terms (e.g. Jardetzky, 1964), one of which is the width of the corresponding peak in the free amino acid. Factors such as the Heisenberg uncertainty in the frequency, and inhomogeneity in the applied magnetic field (e.g. Pople et al., 1959) contribute to the latter width, and make no additional contribution to the protein peak width.

1A(i) Dipolar Interactions

To account for the experimental finding that polymers often have much broader peaks than monomers,

several workers have concluded that dipole-dipole interactions dominate relaxation mechanisms for macromolecules in solution (e.g. Saunders & Wishnia, 1958; Kowalsky, 1962; Markley et al., 1969). The dipole-dipole interaction between two protons makes a contribution (W_d) to the peak width, and this contribution, as derived by Kubo and Tomita (1954), is:-

$$W_d = \frac{1.41 \times 10^{11} \tau_c}{b^6} \left[\frac{3}{5} + \frac{1}{1 + \omega^2 \tau_c^2} + \frac{0.4}{1 + 4\omega^2 \tau_c^2} \right] \qquad (1)$$

where b is the inter-proton distance (°A).

$\omega = 2\pi\nu$ (ν is the radiofrequency e.g. 60 or 100 MHz), and τ_c is the correlation time (sec), i.e. the time required for the molecule to turn through an angle of ca one radian.

This equation is valid provided that:-

(i) rotational motion of the pair of protons is isotropic,

(ii) $\tau_c W_d < 0.01$.

The second condition is easily satisfied for small globular proteins in solution, where $W_d < 100$ Hz, and $\tau_c < 10^{-7}$ sec. The first condition is fulfilled only

by proteins with an approximately spherical shape, or with subunits capable of isotropic rotational motion.

For rotation of a rigid sphere in a viscous medium, Bloembergen et al. (1948) gave:-

$$\tau_c = \frac{4\pi\eta a^3}{3KT} \qquad (2)$$

where η is the viscosity of the solvent,

a is the radius of the sphere,

K is Boltzmann's constant,

and T is the absolute temperature.

For small molecules in non-polar solvents, Pritchard and Richards (1966) obtained better agreement between theory and experiment when a microviscosity factor (M) was introduced:-

$$\tau_c = \frac{4\pi\eta a^3 M}{3KT} \qquad (3)$$

where $M = [6ra^{-1} + (1 + ra^{-1})^{-3}]^{-1}$

and r is the radius of a solvent molecule.

However, the microviscosity factor is approximately unity for protein molecules in water, where $a >> r$.

Inspection of equation (1) shows that a decrease in the internuclear distance, or an increase in the correlation time could broaden a peak. Equation (2) indicates that the value of the correlation time may be

raised by an increase in effective size of a molecule (e.g. by association of rigid molecules).

1A(ii) Exchange

Broadening may also result if a proton exchanges between two different environments at an appropriate rate (e.g. Pople et al., 1959). Consider exchange of protons between two sites, a and b, with fractional populations P_a and P_b, and chemical shifts ν_a and ν_b (Hz) respectively. If the lifetimes at each site, τ_a and τ_b are very large compared with $(2\pi\nu_a - 2\pi\nu_b)^{-1}$, two signals will be observed (Pople et al., 1959). The additional broadening of each peak, due to this exchange, will be:-

$$W_e = (\pi\tau)^{-1} \qquad (4)$$

where $\tau = \tau_a$ or τ_b.

For rapid exchange, τ_a and τ_b are small compared with $(2\pi\nu_a - 2\pi\nu_b)^{-1}$ and a single resonance line is obtained (Pople et al., 1959). Its chemical shift (ν) is given by:-

$$\nu = P_a\nu_a + P_b\nu_b \qquad (5)$$

and its width is given by:-

$$W = P_aW_a + P_bW_b + 4\pi P_a^2P_b^2(\nu_a - \nu_b)^2(\tau_a + \tau_b) \qquad (6)$$

where W_a and W_b are the widths in the absence of exchange.

More complicated expressions result for intermediate rates of exchange where τ_a and τ_b are of the order of $(2\pi\nu_a - 2\pi\nu_b)^{-1}$.

1A(iii) Spin-rotation

Rotational Brownian motion is accompanied by rotation of charges in a molecule, which gives rise to fluctuating magnetic fields. The interaction of these fields with nuclei (spin-rotation interaction) may provide an effective mechanism for relaxation.

In contrast to relaxation by the dipole-dipole interaction, which arises from changes in angular position, variations in angular velocity of the rotating charges are required to produce the fluctuating magnetic fields necessary for relaxation by spin-rotation (Green & Powles, 1965). Consequently, the spin-rotation correlation time (average interval between changes in angular velocity) is distinct from, and usually less than, the correlation time (τ_c) for the dipolar interaction.

As given by Green & Powles (1965), the spin-rotation contribution (W_{sr}) to the peak width is:-

$$W_{sr} = 9.65 \times 10^{52}\ KTIc^2\tau_{sr} \qquad (7)$$

where I is the moment of inertia of the molecule (gm cm^2)

c is the spin-rotation interaction constant (Hz) and τ_{sr} is the spin-rotation correlation time. Provided $\tau_{sr} << \tau_c$, $\tau_{sr} = I(6KT\tau_c)^{-1}$.

Since W_{sr} is proportional to $I^2\tau_c^{-1}$, it may be more significant for large molecules such as proteins, than for small molecules. However, for protons, c is relatively small, and ranges from <1 KHz for benzene to 10 KHz for methane (Pritchard & Richards, 1966).

1A(iv) Miscellaneous

For native proteins, broadening commonly arises from the non-equivalence in magnetic environments for protons which are otherwise identical (McDonald & Phillips, 1969). Any type of side-chain, occurring in several places in the amino-acid sequence, will experience different environments, depending on neighbouring amino acids; hence any peak arising from the side-chains will be composed of a number of separate resonances, each with a slightly different chemical shift.

Further broadening can be produced by the proximity of paramagnetic nuclei, or nuclei with large quadrupolar moments (e.g. Emsley et al., 1965). However, for protons, broadening arising from magnetic anisotropy is negligible (Gutowsky & Woessner, 1956).

1B Denaturation

A protein molecule is said to be denatured when its native conformation has undergone a major change without cleavage of any covalent bond (Joly, 1965; Tanford, 1968).

Typically, a native protein P, is transformed to an unfolded state U, which undergoes further change to give a final product Z.

$$P \rightleftharpoons U \rightleftharpoons Z \qquad (8)$$

More than one conformation may correspond to each of the states P, U, and Z. Furthermore, in the transitions between these states intermediate conformations may occur. If the protein consists of a number of subunits, it may dissociate. Reversible transitions are shown in equation (8), but in many examples of denaturation they are irreversible.

The denatured state U may be a random coil, in which rotation about each bond is as free as rotation about the same bond in a compound of small molecular weight (Tanford, 1968). There are no fixed noncovalent interactions - only transient contacts form. At any instant, a large number of different configurations are present (Flory, 1969). A protein molecule will be described as completely unfolded when it has the same

freedom in configuration as a random coil except for constraints imposed by covalent crosslinks.

More commonly, U may be a partially unfolded structure, which retains fixed noncovalent interactions between amino-acid side-chains.

The denatured product Z may be an aggregate, precipitate, gel, or a refolded non-native conformation. Alternatively, denaturation might not proceed beyond the unfolded state U.

In this thesis we shall be primarily concerned with transitions of the type $P \rightleftharpoons U$.

In a native protein molecule, many side chains are held rigidly, and consequently rotate no faster than the molecule as a whole. On unfolding, however, the gain in freedom of rotation about bonds gives rise to much segmental motion which reduces τ_c and diminishes peak widths (equation (1)).

In equation (8), a distinction between the unfolded and other denatured states was deliberately made, since the NMR spectrum enables us to estimate the extent of unfolding rather than the total extent of denaturation. A spectrum of unfolded molecules is relatively sharp, but the native, refolded or associated states of a protein often have similar spectra with broad resonances.

If the protein is heavily aggregated, however, extremely broad peaks appear, thereby enabling this condition to be recognised.

In a typical isothermal unfolding experiment, known amounts of denaturant were successively added to a protein solution, and the NMR spectrum recorded after each addition.

The extent of unfolding, F, is defined by the relation:-

$$F = \frac{h - h_N}{h_U - h_N} \qquad (9)$$

where h_N is the peak height for native protein,

h_U is the maximum peak height reached in the series

and h is the peak height at a particular concentration of denaturant.

All h's are corrected for dilution effects accompanying addition of denaturant. F is a function of denaturant concentration and this function may vary from one peak to another.

The significance of F is dependent on the nature of the unfolding.

CASE I: If the transition $P \rightleftharpoons U$ is a single step one, with no appreciable concentration of intermediates, and if the exchange between folded and unfolded molecules

is sufficiently slow for W_e to be negligible, then

$$h = \alpha h_U + (1-\alpha)h_N$$

where α is the fraction of unfolded molecules.

$$\text{Then } \alpha = \frac{h - h_N}{h_U - h_N} = F. \qquad (10)$$

In this particular case, F equals the fraction of unfolded molecules.

An example of this behaviour may be found in the unfolding of ribonuclease by urea (Chapter 5). Hollis et al. (1967) used equation (10) to estimate the fraction of unfolded molecules in the thermal denaturation of α-chymotrypsin. However, there was no accompanying evidence to show that the transition was sufficiently slow to apply equation (10).

CASE II: If exchange between the two states of a single-step process is sufficiently rapid, equation (6) becomes

$$W = \alpha W_U + (1-\alpha)W_N$$

where W is the width at half-height of a peak at a particular concentration of denaturant

W_N is the width at half-height of the peak for native protein

and W_U is the width at half-height of the peak for unfolded molecules.

For a Lorentzian peak, $hW = h_U W_U = h_N W_N$.

$$\text{Therefore, } \frac{1}{h} = \frac{\alpha}{h_U} + \frac{(1-\alpha)}{h_N}$$

$$\alpha = \frac{h_U}{h} \frac{(h-h_N)}{(h_U-h_N)}$$

$$= \frac{h_U F}{h} \qquad (11)$$

Hence, throughout the transition, $\alpha > F$, and a plot of F (or h) against denaturant concentration is weighted in favour of the native conformation.

The above treatment would be applicable to the helix-coil transition of synthetic homopolypeptides of high molecular weight, since the interconversion rate is very fast (Bradbury et al., 1969). While the helix-coil transition is multi-state with respect to each molecule (e.g. Flory, 1969) it may, to a first approximation, be regarded as two-state with respect to each side-chain which can exist either in a coil or in a helical region of a particular molecule. Liu et al. (1967) studied the thermal transition for poly-γ-benzyl-L-glutamate in mixtures of benzene and TFA. They reported an apparent anomaly, in that the transition temperature determined by NMR was lower than that obtained by optical rotation. From the NMR peak heights

given by Liu et al. (1967) for the thermal transition in 80% benzene - 20% TFA, F values were calculated according to equation (9) and plotted against temperature in Figure 1.1. Corresponding values of α were estimated from equation (11) and are also shown in Figure 1.1. The mid-point of the F plot is four degrees lower than the mid-point of the α plot (29°C). This explanation partially accounts for the apparent anomaly reported by Liu et al. (1967), but the mid-point of their optical rotation curve was above 30°C.

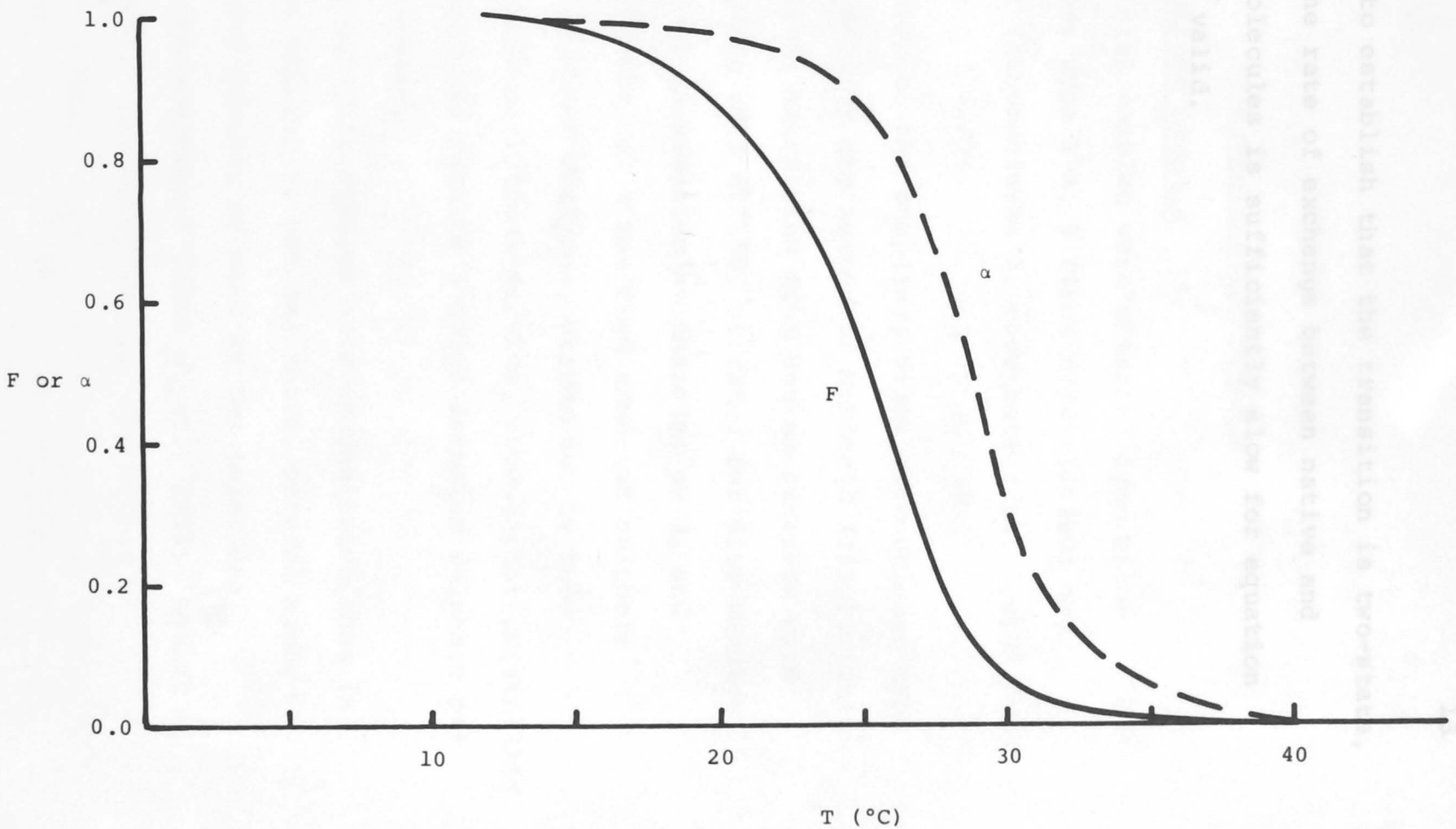
CASE III: If intermediate configurations are present in the transition $P \rightleftharpoons U$, and their peak heights contribute significantly to h, then equation (10) does not hold. Such intermediates may, in some instances, be recognised by (i) the appearance of additional peaks (not arising from either **P** or U) or (ii) the non-equivalence of F values among all peaks in the transition region. An example of (i) is given by the unfolding of ribonuclease by formic acid (Chapter 6) and of (ii) the denaturation of lysozyme by urea (Chapter 8).

Before equating F (the extent of unfolding) and α (the fraction of unfolded molecules) it is therefore

FIGURE 1.1

THERMAL TRANSITION OF POLY-γ-BENZYL-L-GLUTAMATE

F and α derived from Fig. 1 curve A of Liu et al. (1967)



necessary to establish that the transition is two-state, and that the rate of exchange between native and unfolded molecules is sufficiently slow for equation (10) to be valid.

CHAPTER 2

MATERIALS AND METHODS

2A Ribonuclease Samples

Commercial samples were obtained from Sigma (ribonuclease type 1-A, 5 times crystallized) and Worthington (ribonuclease A, phosphate free, lyophilised powder).

According to the supplier, Sigma ribonuclease type 1-A is prepared by the method of McDonald (1955). This method involves extraction from bovine pancreas with 0.25N sulphuric acid at 5°C, stirring for five minutes at 95-100°C in 1M ammonium sulphate at pH 3, and crystallizing several times from ammonium sulphate solutions. Sulphur analyses, carried out by the Australian Microanalytical Service, showed that crystalline Sigma ribonuclease retains 5 moles ammonium sulphate per mole ribonuclease.

Chromatographic studies have demonstrated that in preparations similar to the one above, several minor components are present as well as the major one, designated ribonuclease A (Hirs et al., 1953; Aqvist & Anfinsen, 1959; Taborsky, 1959). The method of

Crestfield et al. (1963) is used by Worthington to prepare ribonuclease A from the crystalline product. The chromatographic and desalting procedures used in their preparation remove sulphate from the protein. This was confirmed by the agreement between the sulphur analysis and the theoretical sulphur content of Worthington ribonuclease A (2.8%).

Although the presence of ammonium sulphate raises the thermal denaturation temperature of ribonuclease, this protein undergoes an unfolding transition at temperatures above 70°C in 1M ammonium sulphate (von Hippel & Wong, 1964). According to the NMR tests for unfolding given in Chapter 3, ribonuclease was extensively unfolded in 1M ammonium sulphate at pH 3 in the temperature range 80° to 100°C. However, there was no evidence for unfolding in the NMR spectrum of ribonuclease in 0.25N sulphuric acid at 5°C.

Since thermal denaturation is involved in all standard methods of preparation (Scheraga & Rupley, 1962) there arises the question of whether the refolded molecules possess the same conformation as ribonuclease in vivo. Until milder methods are developed for preparing ribonuclease, no definite answer can be given. However, the refolded protein possesses enzymatic

activity (e.g. Crestfield et al., 1963) which suggests that the thermal denaturation is reversible. While ribonuclease prepared as above may not necessarily have the same conformation as in vivo, it is commonly referred to as native protein. Its three-dimensional structure has been determined (Kartha et al., 1967) and it has been the subject of numerous other studies (reviewed by Barnard, 1969) the results of which may be compared with NMR data.

2B Other Proteins and Reagents

In addition to ribonuclease, commercial samples of proteins were obtained from the following sources:-

Sigma: insulin (crystalline, bovine pancreas)
thyroglobulin (bovine)

Worthington: trypsin (twice crystallized)
α-chymotrypsin (three times crystallized)
pepsin (twice crystallized)
lysozyme (hen egg-white, twice crystallized)
catalase (beef liver)

Mann: serum albumin (bovine)
fibrinogen (bovine)
myoglobin (sperm whale)

Seravac: myoglobin (whale skeletal muscle)

Gifts of α-lactalbumin B from Mr. K.E. Hopper, and of ovalbumin from Mr. R. Sleigh are gratefully acknowledged.

The above proteins were used without further purification, except that acetate was removed from lysozyme by dialysis against water.

Dichloroacetic acid (M & B laboratory reagent) was distilled under a reduced pressure of nitrogen, and formic acid (analytical reagent) was dried by distillation of a mixture of formic acid and sulphuric acid (Harrap & Woods, 1961). Urea was re-crystallized from water, to remove an impurity giving rise to a peak at 1.05τ.

A mixture of DCl and HCl was obtained by diluting 12M HCl with an equal volume of D_2O. A solution of NaOD was prepared by reacting sodium with D_2O.

Other reagents were the best available and were used without further purification.

2C Spectroscopy

100 MHz spectra were recorded on a Varian HA100 spectrometer, in the Research School of Chemistry, by Mr. A. Arandjelovic. However, the opportunity to have spectra run on this instrument was somewhat limited.

Most of the required spectra were therefore obtained on a 60MHz Perkin-Elmer R10 spectrometer, in the Chemistry Department, School of General Studies. Unless otherwise specified, all 60 MHz spectra were run at 33.4°C using a radiofrequency input setting of 1 mV and a sweep rate of 6.4 Hz per second. A general purpose Digital Equipment PDP-8/S computer was used on-line with the spectrometer to increase the signal-to-noise ratio by averaging spectra (e.g., Ernst, 1965). Peripheral equipment included an ASR-33 teletype, analogue-to-digital converter, real-time clock and a Tektronix storage oscilloscope.

A basic spectra-averaging programme supplied by Digital Equipment was modified, and additional features were incorporated in it.

In order to compensate for magnetic field drift, the computer programme aligned a reference peak in each scan with the same peak in the first scan. Tetramethylsilane served as an internal reference in organic solvents, while in D_2O, the sodium salt of 3-trimethylsilyl-1-propanesulphonic acid was sometimes used. However, as reported previously (Bradbury et al., 1967) this salt interacts with some proteins, and its peak is then broadened. For the purpose of correcting

magnetic field drifts in D_2O, an external reference was therefore used routinely. A capillary tube, containing tetramethylsilane diluted by carbon tetrachloride, was immersed in the NMR sample tube to serve as an external reference. Although it eliminated any possibility of the reference interacting with the sample, this technique had the disadvantage of decreasing the volume of sample being irradiated, and hence decreasing the sensitivity. Chemical shifts are quoted relative to an internal reference.

Correction for baseline drift was also found to be necessary, since at the high amplifier gains used, the spectrum frequently drifted off scale after a few hours. At the end of each scan, the baseline level was corrected by means of a voltage signal from the computer, which actuated an electric motor for a sufficient time to drive a potentiometer to the appropriate setting.

In addition to the above facilities, the computer programme rejected scans which were unsatisfactory because of poor resolution, severe baseline drift, or severe magnetic field drift. The computer was also programmed to estimate chemical shifts, heights, widths, and areas of peaks. Alternatively, areas were determined by cutting out peaks and weighing the paper.

Under programme control, spectra contained in the computer memory could be displayed by the oscilloscope, listed by the teletype, punched out on paper tape, or plotted by the NMR recorder. Spectra stored on punched tape could be read into the computer at any future date, and this was especially useful for obtaining difference spectra. In this technique, the programme aligned reference peaks, and then subtracted one spectrum from another (the spectra could be weighted by appropriate factors to allow for concentration differences).

Another programme was written to estimate peak heights and widths only. At the end of each scan, the programme measured the heights and widths of several prominent peaks that the operator had specified. After a number of scans, the average value of each height and width, together with the variances, could be typed out. This proved more convenient, though no more accurate, than the manual methods used by Bradbury and Stubbs (1968).

Solutions were prepared by dissolving a weighed amount of solid sample in a measured volume of solvent. Unless otherwise specified, all solutions were 10% w/v. When necessary, solutions were adjusted to the required volume in a graduated NMR tube. pH's were measured with

a Beckman Research pH meter. It was fitted with microelectrodes to allow volumes as small as 0.5 ml to be read. Values quoted in this thesis for D_2O solutions are pH meter readings uncorrected for deuterium isotope effects. The relatioship between pH and pD for protein solutions is not well defined (Willumsen, 1968) and the significance of pH values in concentrated urea solutions is in some doubt (Simpson & Kauzmann, 1953; Burk & Greenberg, 1930).

CHAPTER 3

NMR TEST FOR COMPLETE UNFOLDING

3A Introduction

With regard to those examples of denaturation which are predominantly of the type P $\rightleftharpoons$ U, there arises the question of whether U is a cross-linked random coil, or whether it retains non-covalent interactions. Several methods for the determination of molecular weight require proteins to be extensively unfolded e.g. Davison (1968), Parish & Marchalonis (1969). Before applying these methods to uncharacterised proteins, it would be desirable to check that unfolding does occur in the solvent used. In particular, the extent of unfolding in 8M urea is dependent on pH (e.g. Tanford, 1968).

Tanford (1968) has discussed a number of methods e.g. viscometry, optical rotatory dispersion, acid-base titrations, and ultraviolet spectroscopy, which are useful for determining whether or not a denatured protein is completely unfolded. This chapter shows how NMR may be used to detect residual non-covalent interactions in denatured proteins.

McDonald & Phillips (1969) describe a procedure for calculating spectra of completely unfolded proteins in D_2O. The incorporation of amino acids in a polypeptide chain has an effect on their chemical shifts, and in an attempt to allow for this, McDonald & Phillips (1969) modified resonance positions of amino-acid side-chains to force agreement with the actual spectra of heat-denatured lysozyme and ribonuclease in D_2O. These modified resonance positions were then used to calculate other spectra of completely unfolded proteins from their amino-acid compositions. However, Tanford (1968) quotes experimental evidence against globular proteins being completely unfolded by thermal denaturation. Notably, a further change in optical rotation can be induced in heat-denatured lysozyme and ribonuclease by the addition of GuCl (Aune et al., 1967). The poor agreement between some of the calculated and observed spectra (McDonald & Phillips, 1969) can be attributed to incomplete unfolding of heat-denatured proteins.

Because of the difficulty in allowing theoretically for solvent effects, and for effects caused by the incorporation of amino-acid residues in polypeptide chains, an empirical approach is adopted in the next section.

3B NMR Spectra of Unfolded Proteins

Unfolded protein molecules give rise to sharper and better resolved spectra than native proteins e.g. Bovey et al. (1959). This is illustrated in Figure 3.1, which shows spectra of native ribonuclease in D_2O at pH 4.7, and of unfolded ribonuclease in d-TFA. Throughout this thesis, peaks will be referred to by the names used in Figures 3.1 and 5.2. Peak assignments in Figure 3.1 are based on those of Bovey et al. (1959), Kowalsky (1964) and Mandel (1965). For amino acids, a detailed list of chemical shifts in TFA has been given by Bak et al. (1968) and in D_2O by McDonald & Phillips (1969).

In addition to TFA, it is well established that most synthetic polypeptides and proteins are unfolded in FA (e.g. Harrap & Woods, 1961) and DCA (e.g. Doty et al., 1956) as well as in 6M GuCl and 8M urea at certain pH's (e.g. Tanford, 1968). To obtain spectra of unfolded polypeptides, a number of proteins (listed in Table 3.1) were examined in these denaturants. Spectra of 10% solutions were accumulated for ca 3 hours (100 scans) on the 60 MHz spectrometer. To minimise alteration of covalent bonds, spectra were recorded as soon as possible after dissolution.

Listed below are the peaks named in Figure 3.1, together with the protons giving rise to each peak.

C-2 HIS	C-2 ($\varepsilon 1$) imidazole proton of histidine
C-4 HIS	C-4 ($\delta 2$) imidazole proton of histidine
PHE	aromatic protons of phenylalanine
TYR	aromatic protons of tyrosine
α-CH	peptide protons on α-carbon atoms
HDO	solvent protons
S	spinning side-band of HDO
MET	S-CH_3 protons of methionine
ALA	methyl protons of alanine
THR	methyl protons of threonine
METHYL	methyl protons of isoleucine, leucine and valine
TMS	methyl protons of tetramethylsilane or sodium 3-trimethylsilyl-1-propanesulphonate

Most resonances which are not named arise from methylene protons.

FIGURE 3.1

60 MHZ SPECTRA OF SIGMA RIBONUCLEASE

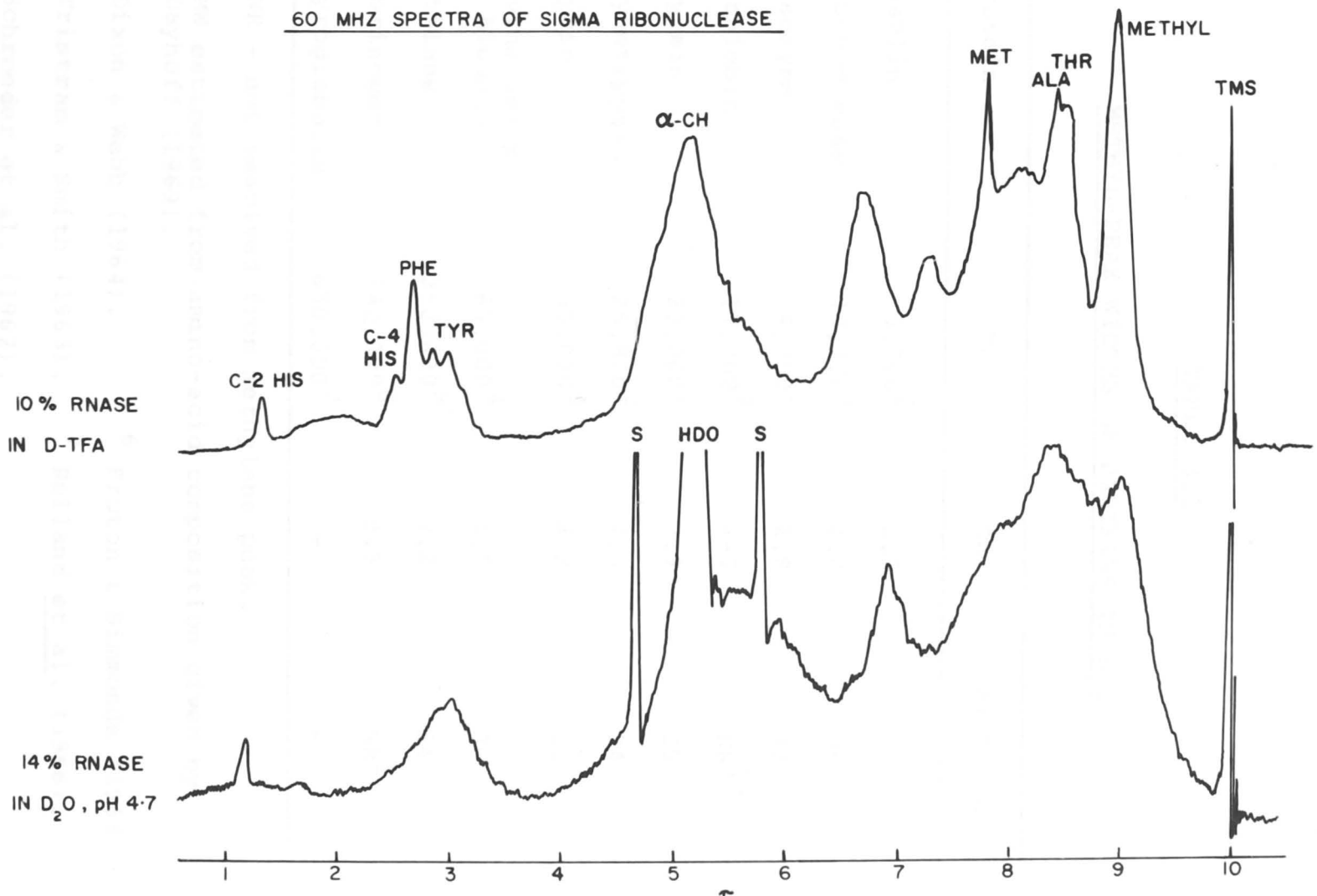


TABLE 3.1

METHYL-PEAK WIDTHS OF PROTEINS IN D_2O

Protein	MW	pH	Width (Hz)
Insulin	5,700[2]	2.9	54
Ribonuclease	13,700[2]	4.0	36
Lysozyme	14,300[2]	2.8	42
Myoglobin	17,200[2]	4-7	NR[1]
Trypsin	23,300[2]	3.9	25
Chymotrypsin	25,800[2]	4.0	34
Pepsin	35,000[3]	4.2	23
Bovine Serum Albumin	65,000[4]	4.0	36
Catalase	250,000[5]	7.2	54
Fibrinogen	340,000[6]	9.5	NR[1]
Thyroglobulin	650,000[7]	-	-

[1] NR - not resolved from methylene peak.

[2] MW estimated from amino-acid composition given by Dayhoff (1969).

[3] Dixon & Webb (1964).

[4] Tristram & Smith (1963).

[5] Schroeder *et al*. (1962).

[6] Fruton & Simmonds (1958)

[7] Rolland *et al*. (1966).

In addition to unfolding, catalase and thyroglobulin dissociate into subunits (Reithel, 1963). For solutions of insulin in TFA, the slow formation of $-OCOCF_3$ and $-NHCOCF_3$ groups, together with their effect on biological activity, has been described by Bak et al. (1967). In FA, formylation of serine and threonine residues occurs (e.g. Narita, 1959). This caused a peak due to the β-carbon protons of serine and threonine to shift from 5.85τ to 5.10τ, after storage of an ovalbumin solution for one day at 33.4°C. However, no other changes in the spectrum were observed.

As shown in Figure 3.1 for ribonuclease, the methyl peak, arising from the methyl groups of isoleucine, leucine and valine, is usually the most prominent peak in a protein spectrum. At 60 MHz this composite peak is not resolved into its constituent resonances, but appears as a single band. For the series of proteins and denaturants investigated, the widths of the methyl peak are shown in Table 3.2.

In addition, the methyl-peak widths for several concentrations of ribonuclease in formic acid are given in Table 3.3.

Table 3.3 shows no significant variation in width with protein concentration. This property is

TABLE 3.2

METHYL-PEAK WIDTHS FOR UNFOLDED PROTEINS

Compound	Width (Hz)				
	d-TFA	FA	DCA	6M GuCl[1]	8M Urea[1]
L-Leu	8	7	8	8	8
poly-L-Leu	10	insol	insol	insol	insol
L-Leu, L-Val, and L-Ileu[2]	14	15	17	12	11
Insulin	14	13	17	23	36
Ribonuclease	16	14	23	14	14
Lysozyme	16	13	23	15	14[3]
Myoglobin	14	15	19	14	17
Trypsin	16	14	17	16	16
Chymotrypsin	14	13	17	14	14
Pepsin	14	13	18	14	20[4]
Bovine Serum Albumin	14	14	19	16	14
Catalase	15	14	26	16	17
Fibrinogen	16	16	29	17	23
Thyroglobulin	15	13	22	18	18
Mean of Proteins	15	14	21	16	18

[1] pH 4-5, except where otherwise noted

[2] equi-molar mixture

[3] pH 2.8

[4] pH 3.2

TABLE 3.3

METHYL-PEAK WIDTHS FOR RIBONUCLEASE IN ANHYDROUS FORMIC ACID

Protein Concentration (% w/v)	Width (Hz)
1	13
5	14
10	14
20	14

characteristic of synthetic polypeptides in the random-coil configuration (Bradbury & Stubbs, 1968). Furthermore, the widths in Table 3.2 are not dependent on molecular weight for any of the unfolding solvents used. This lack of dependence on molecular weight is also characteristic of random-coil polymers (Bradbury & Stubbs, 1968; Chapman, 1968).

Of the factors mentioned in Chapter 1, the restricted mobility of amino-acid side-chains, and the nonequivalent magnetic environments for otherwise identical protons, are primarily responsible for the

relatively broad peaks exhibited by native proteins in solution (McDonald & Phillips, 1969). Both effects arise from the existence of fixed non-covalent interactions between amino-acid side-chains in native proteins. During unfolding, these interactions are removed, and the peaks become narrower. Consequently, the methyl-peak widths in native proteins (Table 3.1) are much larger than the widths for unfolded proteins in Table 3.2. Furthermore, the smallest widths in Table 3.2 are associated with completely unfolded proteins. In d-TFA and FA, the widths are all less than 17 Hz, and are not significantly greater than the width observed for a mixture of the free amino acids.

Average chemical shifts and widths for the C-2 histidine and methionine peaks are shown in Table 3.4. Except for those spectra where the relevant peak was missing, all proteins in Table 3.1 were included in each average.

For every solvent, all chemical shifts, for each of the C-2 histidine and methionine peaks, were within 0.04τ of the relevant mean in Table 3.4. The constancy of the chemical shifts for unfolded proteins may be contrasted with their variability in spectra of native proteins. For example, in lysozyme spectra at low pH

in D_2O, the C-2 peak of His 15 is at 0.95τ, whereas the corresponding peak of His 48 in ribonuclease A is at 1.75τ. Each deviation in chemical shift is due to the influence of neighbouring side-chains in the three-dimensional structure of the native protein. When the protein is unfolded, their influence is replaced by solvent effects, and the chemical shifts revert to those listed in Table 3.4.

TABLE 3.4

C-2 HISTIDINE AND METHIONINE PEAKS IN UNFOLDED PROTEINS

	Average Chemical Shift (τ)		Average Width (Hz)	
Peak	C-2 His	Met	C-2 His	Met
d-TFA	1.36	7.84	5	4
FA	-[1]	7.88	-[1]	3
DCA	1.32	7.89	5	5
6M GuCl[2]	1.23	7.89	4	4
8M Urea[2]	1.38	7.91	5	4

[1] Obscured by solvent peak.

[2] pH 4-5.

One of the most pronounced solvent effects is associated with the α-CH peak, which is 0.17 ppm further downfield in d-TFA than in FA. This is most likely due to additional protonation of the backbone peptide groups in d-TFA (Bradbury & Fenn, 1969). Unfortunately, the α-CH peak could not be observed in DCA or aqueous solvents, because of overlapping solvent peaks.

3C Examination of Spectra for Residual Non-covalent Interactions

Any significant difference in chemical shift or width of a peak, from that given in Table 3.2 and 3.4 for completely unfolded proteins, may be taken as evidence for the presence of non-covalent interactions. For the proteins and denaturants examined, such differences for the C-2 histidine, methionine and methyl peaks are listed in Table 3.5. As an extreme case of broadening, a diminished peak area may also be indicative of incomplete unfolding. Using the area of the methyl peak as a reference, the number of protons contributing to each of the C-2 histidine, aromatic and methionine peaks, was estimated from the amino-acid composition of the protein, and from the area of the relevant peak. Peaks lacking a significant proportion (ca 0.3) of the

TABLE 3.5

DEVIATIONS IN HISTIDINE, METHIONINE, METHYL AND AROMATIC PEAKS FOR UNFOLDED PROTEINS

Protein	Deviation				
	d-TFA	FA	DCA	6M GuCl[1]	8M Urea[1]
Insulin	-	-	-	Broad methyl	Broad methyl
Ribonuclease	-	-	Broad methyl	-	-
Lysozyme	Small aromatic	-	Broad methyl	-	Broad methyl
Myoglobin	Methionine not visible	Small methionine	-	Small methionine	Small methionine
Trypsin	-	-	-	Double methionine	Double methionine
Chymotrypsin	-	-	-	-	-
Pepsin	-	-	-	-	Small[2] methionine Broad methyl
Bovine Serum Albumin	-	-	-	-	-
Catalase	Small C-2 histidine Small aromatic	-	C-2 histidine not visible Broad methyl	Small C-2 histidine	Small C-2 histidine
Fibrinogen	-	-	C-2 histidine not visible Broad methyl	-	Broad methyl
Thyroglobulin	Small aromatic	-	C-2 histidine not visible Broad methyl	-	-

[1] pH 4-5

[2] pH 3.2

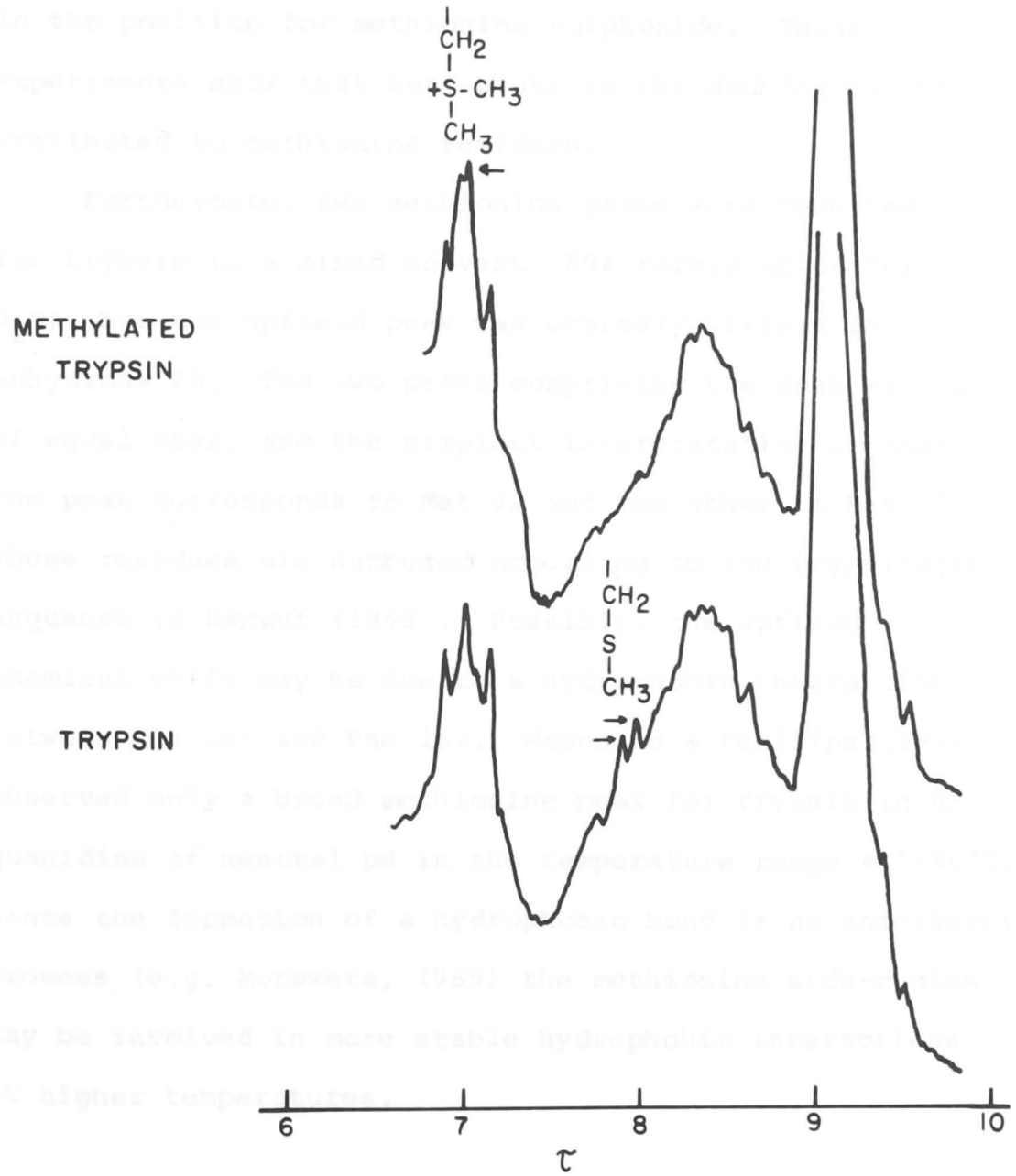
theoretical area are mentioned in Table 3.5. Amino-acid compositions were obtained from Dayhoff (1969) for the first six proteins in Table 3.5. Amino-acid analyses for pepsin and bovine serum albumin were taken from Tristram & Smith (1963), catalase from Schroeder et al. (1962), fibrinogen from Mihalyi et al. (1964) and thyroglobulin from Rolland et al. (1966).

3C (i) Chemical Shifts

As shown in Table 3.5, the only significant deviations in chemical shift are associated with the double peaks observed in the methionine region for trypsin in 6M GuCl and 8M urea. In each case, one of these peaks was in the position for a completely unfolded methionine residue, while the other was upfield by 0.05τ. The absence of the upfield peak in the TFA spectrum, shows that no constraint imposed by the sequence is responsible for the abnormal chemical shift. In addition to being present at pH 4.7, both peaks were also observed at pH 8.0 in 6M GuCl. Methylation, by saturating with methyl iodide and stirring overnight, reduced the size of the double peak in the spectrum at pH 8.0; a new peak appeared at 7.02τ, in the position for the methyl peak of S-methyl methionine (Figure 3.2).

FIGURE 3.2

60 MHZ NMR SPECTRA OF TRYPSIN IN 6M GuCl (pH 8·0)



Similarly, when 0.1 ml 30% hydrogen peroxide was added to 0.5 ml trypsin in 6M GuCl at pH 8.0, the doublet almost disappeared and was replaced by a peak at 7.22τ in the position for methionine sulphoxide. These experiments show that both peaks in the doublet can be attributed to methionine residues.

Furthermore, two methionine peaks were observed for trypsin in a mixed solvent (80% formic acid- 20% D_2O), but the upfield peak was scarcely visible in anhydrous FA. The two peaks comprising the doublet are of equal area, and the simplest interpretation is that one peak corresponds to Met 92 and the other to Met 166. These residues are numbered according to the trypsinogen sequence in Dayhoff (1969). Possibly, the upfield chemical shift may be due to a hydrophobic interaction between Met 166 and Phe 167. McDonald & Phillips (1969) observed only a broad methionine peak for trypsin in 6M guanidine at neutral pH in the temperature range 40°-80°C. Since the formation of a hydrophobic bond is an endothermic process (e.g. Morawetz, 1965) the methionine side-chains may be involved in more stable hydrophobic interactions at higher temperatures.

An upfield methionine peak also appears as an intermediate stage in the progressive unfolding of ribonuclease by increasing concentrations of FA or potassium thiocyanate (Chapters 6 and 7).

3C (ii) Widths

Although in d-TFA and FA, the protein methyl-peak widths are all less than 17 Hz (Table 3.2), some of these widths are considerably larger in DCA, 6M GuCl and 8M urea (Table 3.5). Such broadening indicates retention of non-covalent interactions between amino-acid side-chains. For example, the broad methyl peak of insulin reflects the well-known association of this protein in urea solutions (Reithel, 1963). Again, the broad methyl peak for pepsin in 8M urea confirms the incomplete unfolding reported by Blumenfeld et al. (1960).

3C (iii) Areas

Several examples of diminished peak areas, and of peaks too broad to be visible, are listed in Table 3.5. As mentioned in Section 3B, reduction in apparent area is considered to be an extreme case of peak broadening, for some or all of the resonances contributing to the peak. For both samples of myoglobin (Mann and Seravac) the methionine peak was absent in the d-TFA spectrum,

but was present with its expected area in the DCA spectrum. Residual ordered structure may therefore be retained even when a protein is unfolded by such a powerful denaturant as d-TFA. Whatever the denaturant being used, this emphasises the need to check that residual structure is absent when an uncharacterised protein is required in a completely unfolded state e.g. for chemical modification or for molecular weight determination by gel filtration.

3D Applications of the NMR Test for Complete Unfolding

The NMR test for complete unfolding consists simply in examining the protein spectrum for abnormal chemical shifts and excessive widths. Inspection of the width of the methyl peak is sometimes sufficient for a rough indication of whether or not a protein is extensively unfolded in a particular solvent. Because of the prominence of this peak in most protein spectra, a single scan of the spectrum (five minutes) may be adequate for a 10% protein solution, but accuracy can always be improved by averaging a number of scans.

One advantage of using NMR is that the types of side-chains involved in interactions are detected. While acid-base titrations give information on titratable

groups, and ultraviolet spectroscopy is useful for studying aromatic side-chains, only NMR yields data on the methyl groups of methionine, isoleucine, leucine and valine, as well as the other groups.

In addition to offering a qualitative test for complete unfolding, NMR provides a quantitative method for estimating the extent of unfolding, by means of equation (9).

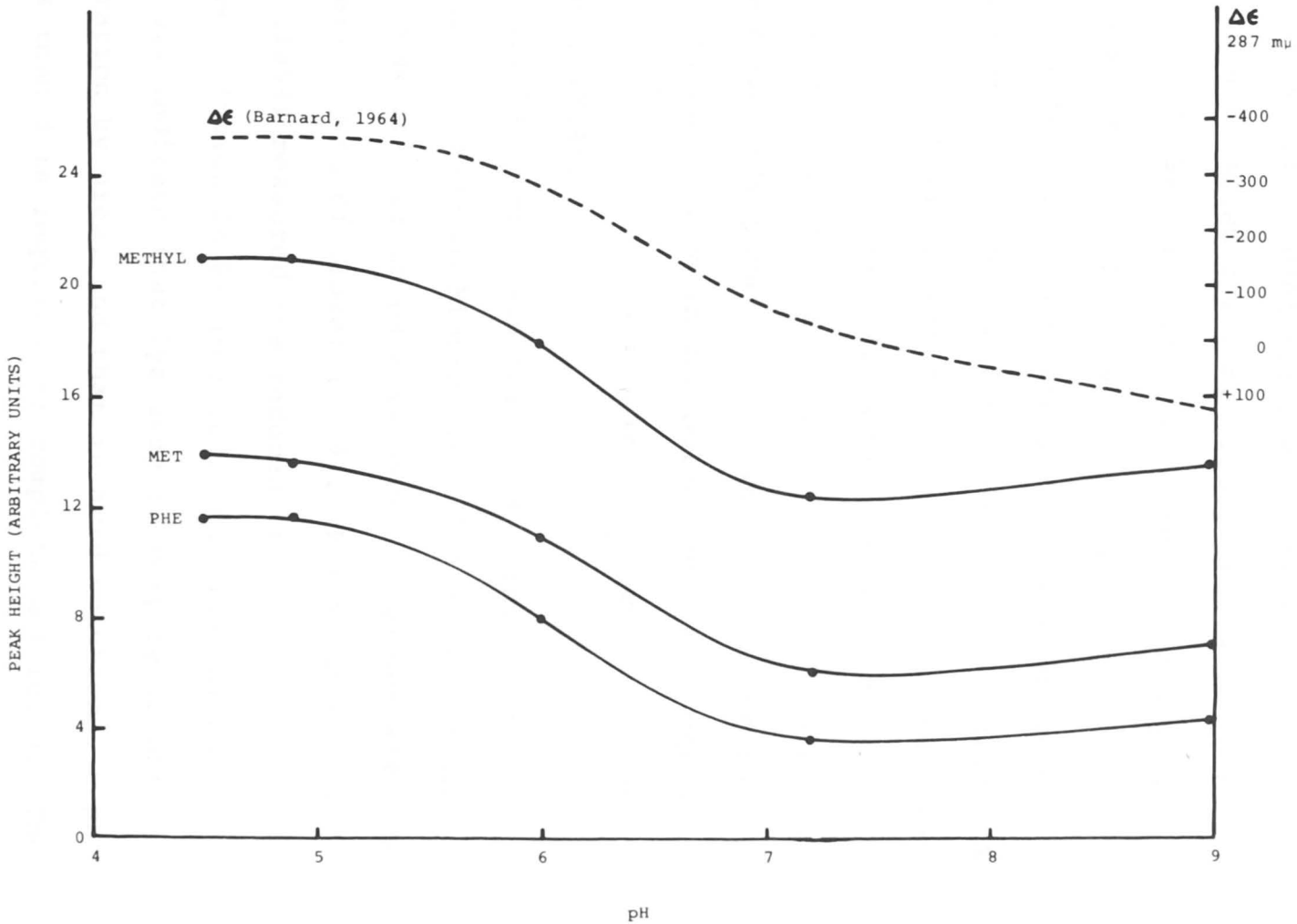
3D (i) Ribonuclease and Lysozyme

For ribonuclease and lysozyme in 8M urea, the variation in unfolding with pH has been extensively studied by a variety of methods (viscometry, ultraviolet spectroscopy and gel filtration) in several laboratories (Glazer, 1959; Barnard, 1964; O'Shea, 1970). These results can now be compared with those obtained by NMR methods.

In the spectrum of native ribonuclease (Figure 3.1) the methionine and phenylalanine peaks are not visible, while the methyl peak is not sharp. In contrast, all three peaks are prominent in the spectrum of unfolded ribonuclease. The height (or width) of a peak may be taken as an indication of the extent of unfolding. In Figure 3.3, peak heights are plotted against pH for a

FIGURE 3.3

RIBONUCLEASE IN 8M UREA

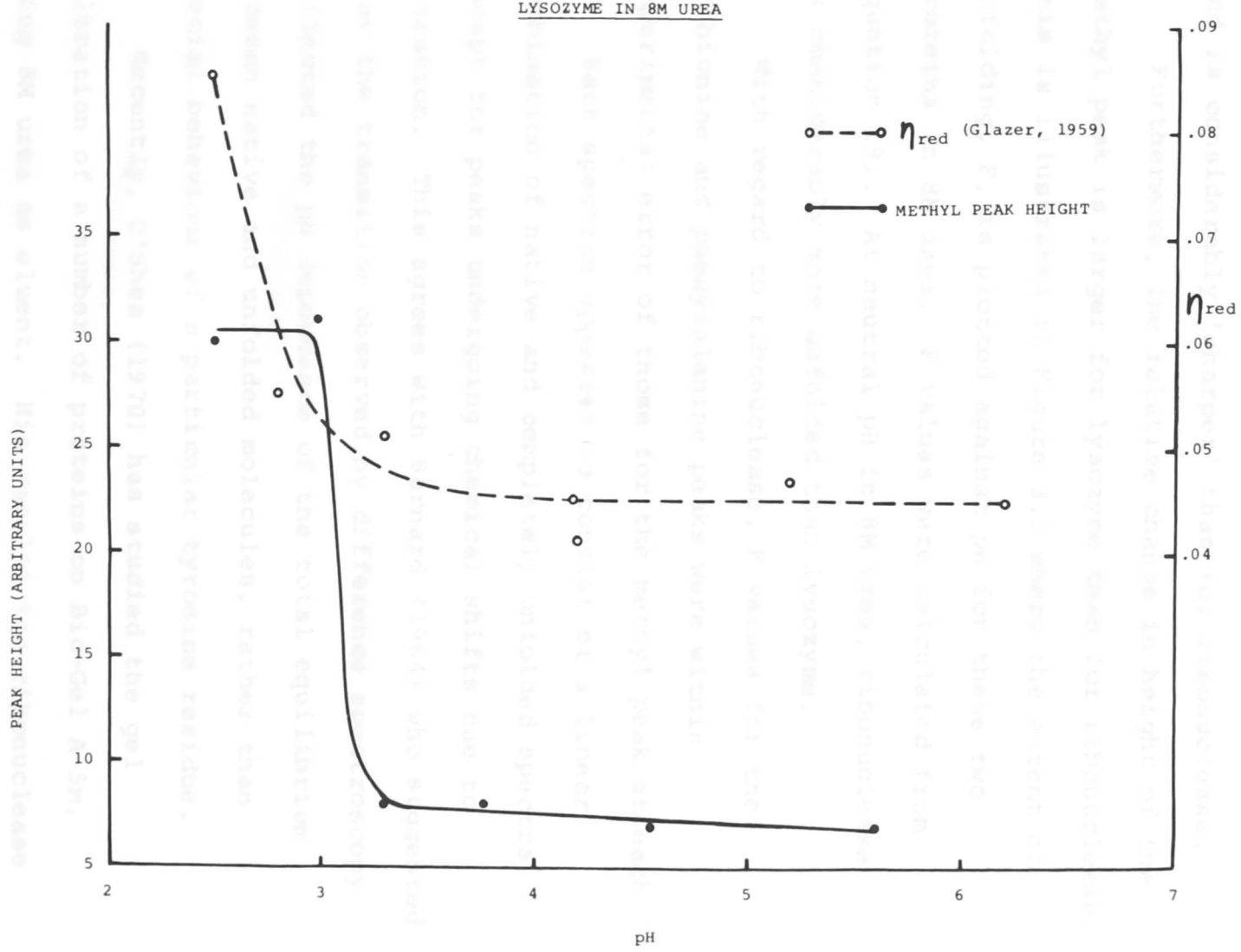


10% solution of ribonuclease in 8M urea. The curve obtained by ultraviolet difference spectroscopy (Barnard, 1964) is also shown for comparison. For a range of pH's, at 25°C Barnard (1964) measured the change in molar absorbance ($\Delta\varepsilon$) at 287 mμ, in passing from the initial state (i.e. immediately after mixing protein and urea solution) to the final steady-state value. At each pH, the final concentration of ribonuclease A was 0.2-0.3%, and of urea, 8M. A transition in the pH range 5.5 to 7.2 is clearly shown by all curves which are approximately parallel at acid pH's. Above pH 7.5, the additional change in the molar absorbance marks the beginning of the tyrosyl ionisation spectral change (Barnard, 1964).

A similar comparison for a 10% solution of lysozyme in 8M urea is made in Figure 3.4. The height of the methyl peak at various pH's is shown, together with the viscometric data of Glazer (1959). For a range of pH's, Glazer (1959) measured the reduced viscosity of a 0.5% lysozyme solution in 8M urea at 30°C. The curves in Figure 3.4 indicate that lysozyme is very resistant to denaturation by urea, and that in acid solutions, a pH of less than 3 is required for complete unfolding. The

FIGURE 3.4

LYSOZYME IN 8M UREA



transition occurs over a pH range of less than one unit, and is considerably 'sharper' than for ribonuclease.

Furthermore, the relative change in height of the methyl peak is larger for lysozyme than for ribonuclease. This is illustrated in Figure 3.5 where the extent of unfolding, F, is plotted against pH for these two proteins in 8M urea. F values were calculated from equation (9). At neutral pH in 8M urea, ribonuclease is considerably more unfolded than lysozyme.

With regard to ribonuclease, F values for the methionine and phenylalanine peaks were within experimental error of those for the methyl peak at each pH. Each spectrum appeared to consist of a linear combination of native and completely unfolded spectra, except for peaks undergoing chemical shifts due to titration. This agrees with Barnard (1964) who suggested that the transition observed by difference spectroscopy reflected the pH dependence of the total equilibrium between native and unfolded molecules, rather than special behaviour of a particular tyrosine residue.

Recently, O'Shea (1970) has studied the gel filtration of a number of proteins on Bio-Gel A-5m, using 8M urea as eluent. His results for ribonuclease and lysozyme are summarised in Table 3.6.

FIGURE 3.5

DEPENDENCE OF F ON pH

RIBONUCLEASE & LYSOZYME IN 8M UREA

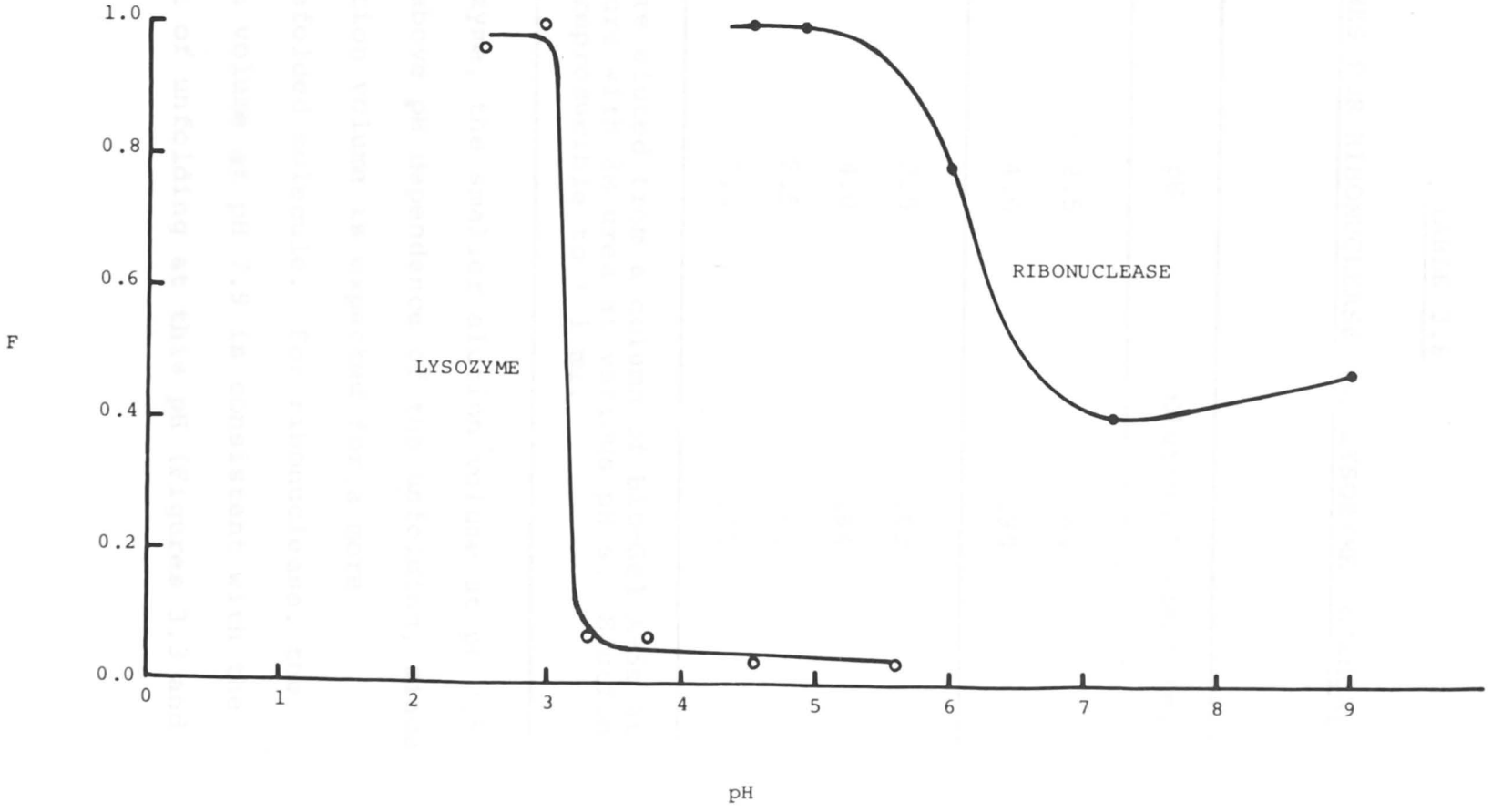


TABLE 3.6

ELUTION VOLUMES FOR RIBONUCLEASE AND LYSOZYME (O'SHEA, 1970)

Protein	pH	Elution Volume* (ml)
Lysozyme	2.5	164
	4.0	190
Ribonuclease	2.5	163
	4.0	164
	5.5	162
	7.9	173

* Proteins were eluted from a column of Bio-Gel A-5m at room temperature with 8M urea at various pH's. Elution volumes were reproducible to ± 1 ml.

For lysozyme, the smaller elution volume at pH 2.5 confirms the above pH dependence of the unfolding, since a smaller elution volume is expected for a more extensively unfolded molecule. For ribonuclease, the larger elution volume at pH 7.9 is consistent with the reduced extent of unfolding at this pH (Figures 3.3 and 3.5).

Transition curves determined by NMR in this section are therefore in agreement with results obtained by other methods.

3D (ii) S-carboxymethyl Proteins from Kangaroo Fur

As an example of the application of the NMR test for unfolding to a relatively uncharacterised protein, spectra of kangaroo fur proteins in 8M urea at different pH's were examined.

A mixture of high-sulphur proteins, from kangaroo fur, was kindly supplied by Dr. J.M. O'Shea. The preparation of these proteins involved cleavage of the disulphide bonds with tri-n-butylphosphine, and reaction with iodoacetic acid to produce S-carboxymethylcysteinyl residues (O'Shea, 1970). The methylene protons of the S-carboxymethyl group give rise to a sharp peak at 6.7τ in unfolded molecules, as shown in Figure 3.6. This peak will be referred to as the SCM peak.

Figure 3.7 shows the variation in height of the methyl, threonine and SCM peaks with pH in 8M urea. Although the methyl and threonine peaks indicate partial unfolding of these proteins at pH <4, the SCM peak does not appear until the pH is raised above 6. All peaks show that maximum mobility of side-chains is exhibited

FIGURE 3.6

HIGH-SULPHUR PROTEINS FROM KANGAROO FUR (60 MHZ)

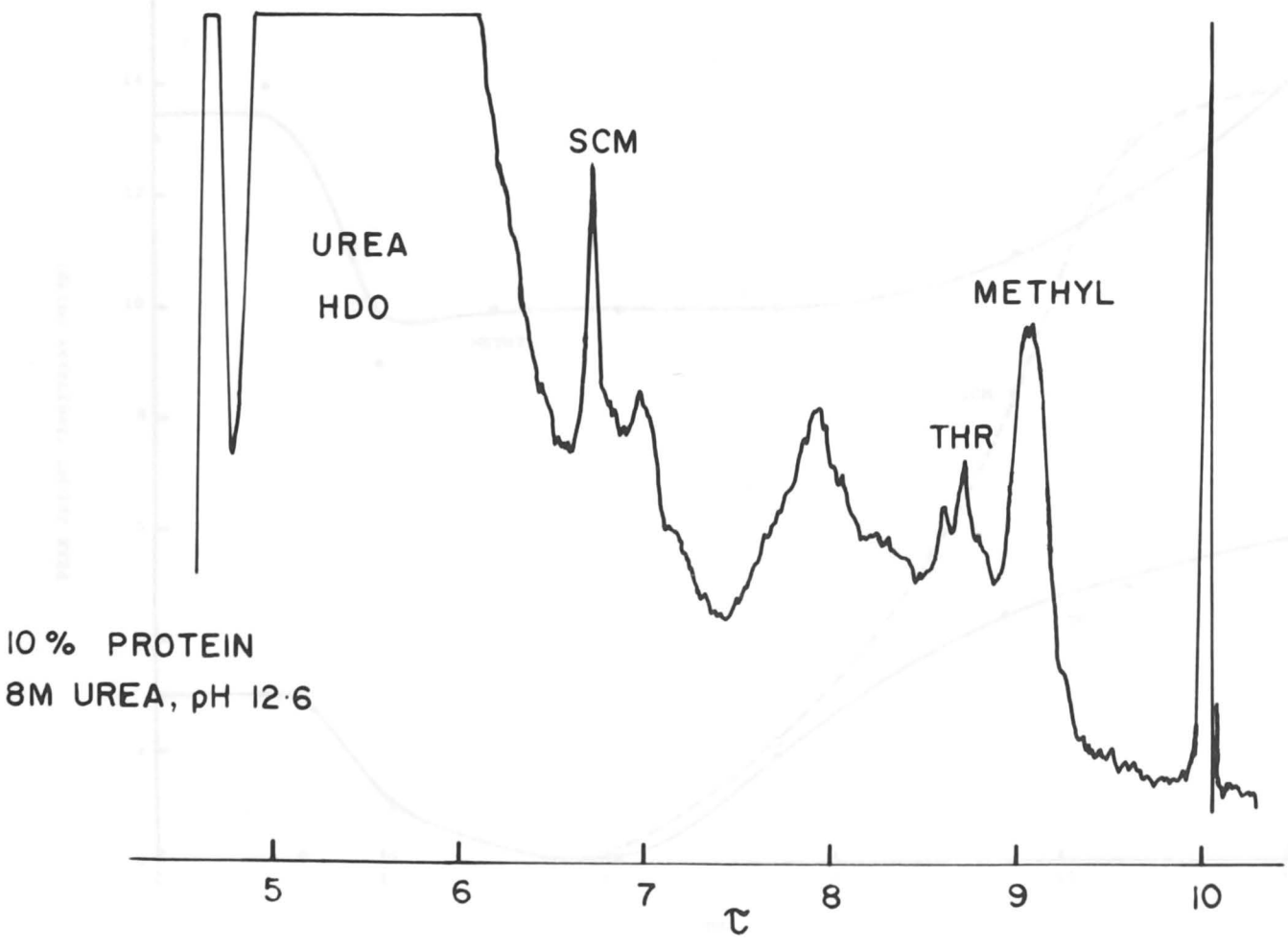
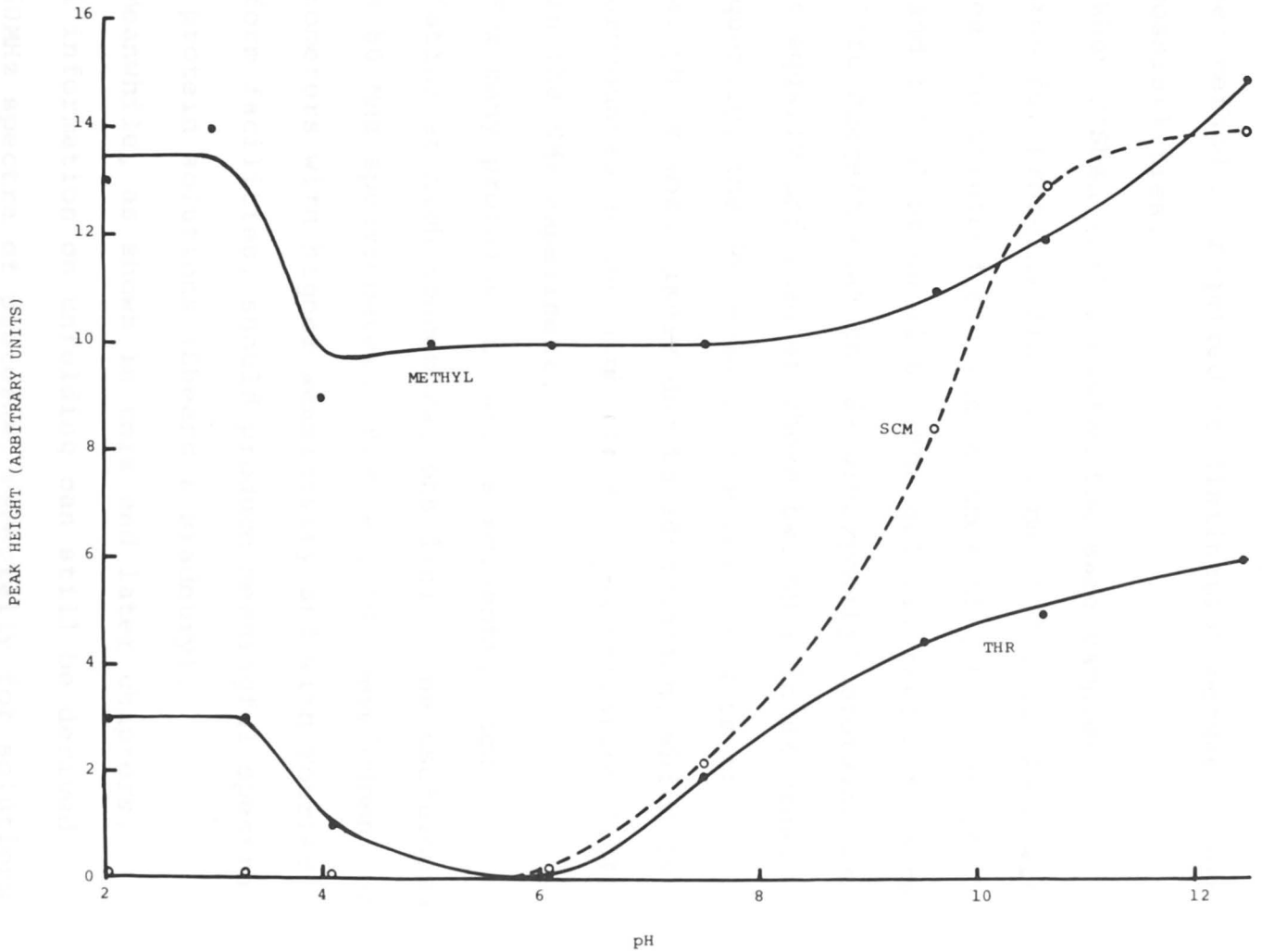


FIGURE 3.7

HIGH-SULPHUR PROTEINS FROM KANGAROO FUR IN 8M UREA



at high pH values. The smaller peak heights at neutral pH indicate association and/or incomplete unfolding. Another method is required to distinguish between these two possibilities.

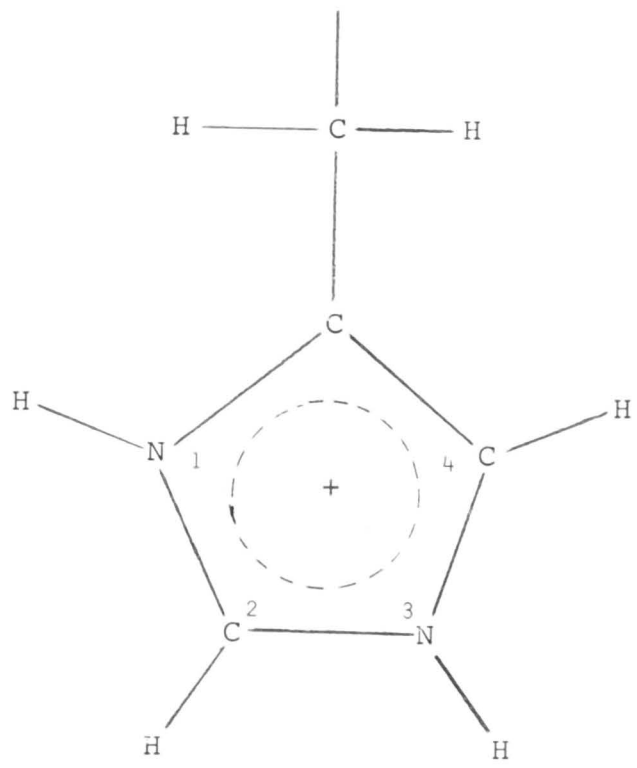
When O'Shea (1970) eluted the same samples of kangaroo fur proteins from a column of Bio-Gel A-5m with 8M urea, he obtained elution volumes of 136 ml at pH 8.0, and 139 ml at pH 11.0. The gel filtration results therefore suggest that the S-carboxymethyl proteins are almost equally unfolded at these two pH's in 8M urea. Consequently, the decrease in NMR peak heights at neutral pH is most likely due to association, which is more pronounced at the high protein concentration (10%) used in the NMR experiment.

For many proteins in various solvents, such association at high concentrations limits the usefulness of the 60 MHz spectrometer. However, the development of spectrometers with higher sensitivity and with Fourier Transform facilities, should produce meaningful spectra of 1% protein solutions (Sheard & Bradbury).

Meanwhile, as shown in this and later chapters, useful information on unfolding can still be derived from 60MHz spectra of proteins, especially for solutions which are substantially free of association.

CHAPTER 4

HISTIDINE PEAKS IN PROTEIN SPECTRA



4A Introduction

Because of their presence at the active sites of several enzymes, histidine residues continue to attract special interest from protein chemists. The histidine imidazole group is particularly amenable to study by NMR, since its C-2 and C-4 peaks can be readily separated from other resonances.

Kowalsky (1962) reported the presence of an imidazole peak in a 60 MHz spectrum of ribonuclease unfolded by 8M urea. He was able to distinguish this C-2 peak from overlapping NH resonances, since protons bonded to nitrogen atoms readily exchange with solvent deuterons at 60°C (Kowalsky, 1964). The chemical shift of the C-2 peak in heat-denatured ribonuclease was found to be dependent on pH, and this confirmed its assignment. Working with native ribonuclease A in D_2O, Bradbury and Scheraga (1966) observed three C-2 histidine peaks at pH's in the imidazole titration range. From each plot of chemical shift against pH, an apparent pK was obtained. Meadows et al. (1967) repeated this titration but used 0.2M sodium deutero-acetate as the solvent. In this buffer solution, at 100 MHz, four C-2 peaks were resolved. Each peak corresponded to one of the four histidine residues in ribonuclease. Meadows et al. (1968) claim to have assigned these peaks to the corresponding residues in the enzyme, which contains two histidines (12 and 119) at the active site.

While suggestive, their evidence is by no means conclusive. One C-2 peak, with pK 6.4, was 0.5 ppm upfield from the others at low pH. This peak was assigned to His 48, a residue which is "buried" in the

crystalline structure of ribonuclease A (Kartha et al., 1967). Further support was derived from studies on ribonuclease S*, where His 48 is less "buried" (Wyckoff et al., 1967) and the chemical shift of its C-2 peak is closer to normal (Meadows et al., 1968). However, there is no well-defined relationship between "buriedness" and chemical shift. Although the nearby residues Asp 14 (Ruterjans et al., 1969) and Tyr 25 (Meadows et al., 1969) may affect the chemical shift of the C-2 peak of His 48, no quantitative estimates of their effects have been reported. Carboxymethylation of either His 12 or His 119 altered the pK's of two imidazole groups, neither of which had been assigned to His 48. The fourth peak was thus assigned to His 105. In order to assign the remaining two peaks, Meadows et al. (1968) titrated two samples of ribonuclease S, in one of which the C-2 proton of His 12 was replaced by a deuteron. The spectrum of the deuterated sample lacked one C-2 peak, and this peak was assigned to His 12 with a pK of 6.7. The remaining peak corresponded to His 119 with a pK of 6.3. This completes the assignment for

---

* Ribonuclease S has the same primary structure as ribonuclease A, except that the peptide bond between Ala 20 and Ser 21 is split (Richards & Vithayathil, 1959).

ribonuclease S. However, for ribonuclease A, the final assignment given by Meadows et al. (1968 & 1969) is dependent on the implicit assumption that the residue with pK of 6.3 in ribonuclease S, corresponds to the one with pK 5.8 in ribonuclease A (His 119). While such an assumption might appear reasonable, further evidence is required to confirm this assignment. Meanwhile, the tentative assignment of Meadows et al. (1968 & 1969) will be adopted in this thesis.

Subsequent titration studies of ribonuclease A concerned its interaction with inhibitors (Meadows & Jardetzky, 1968; Ruterjans & Witzel, 1969) as well as the effect of temperature and salt concentration (Roberts et al., 1969a). A modified mechanism for the enzymic action of ribonuclease has recently been proposed by Roberts et al. (1969b) on the basis of their latest NMR data, together with the X-ray data of Kartha et al. (1967) and Wyckoff et al. (1967).

The titration of histidine residues in other proteins has also been followed by NMR. Bradbury & Wilairat (1967) determined an apparent pK (5.8) for the single imidazole in hen egg-white lysozyme, while Meadows et al. (1967) and Cohen (1969) found that in human lysozyme, the dissociation constant was much higher

(>7). Apparent pK's for the imidazole groups of staphylococcal nuclease (Meadows et al., 1967) and ribonuclease T_1 (Ruterjans et al., 1969) have been reported, but in the latter case, there was poor agreement with values obtained by other methods (Iida & Ooi, 1969). A pK determined by NMR may be in error if conformational changes alter the chemical shift in the titration range.

The bulk of the results so far have come from observations on the C-2 peaks, the C-4 resonances being neglected in most studies. In ribonuclease A, the C-4 peak of His 105 was the only one observed by Meadows et al. (1967). A method for distinguishing the other three C-4 imidazole peaks from overlapping aromatic resonances of phenylalanine and tyrosine, is described in section 4B.

For some proteins, throughout wide ranges of pH, not all of the C-2 histidine resonances are visible. Bradbury and Wilairat (1967) could not detect any histidine peaks in native trypsin or chymotrypsin, although they were present when these enzymes were unfolded. This was not necessarily due to their high molecular weights, since Meadows et al. (1968) observed an extremely broad C-2 peak for His 12 in the S-peptide

(residues 1-20) of ribonuclease. Broadening of imidazole peaks in protein spectra has, in the past, been attributed to a lack of mobility, or to exchange between different environments (e.g. Bradbury & Wilairat, 1967; Meadows & Jardetzky, 1968). Another source of broadening, arising from proximity of protons, is discussed in section 4C.

4B Ribonuclease Imidazole Titrations

4B (i) Experimental

10% solutions of ribonuclease in D_2O were adjusted to the required pH by the addition of 6M DCl-HCl or 2M NaOD. After the addition of acid or base, several minutes were allowed for equilibration. pH readings were checked at the end of every spectrum accumulation. At each pH, 30 sweeps were accumulated at 100 MHz, using a sweep rate of 5 Hz per second. Spectra were recorded on punched tape, for subsequent determination of difference spectra, as described in section 2C. Following Bradbury & Scheraga (1966), chemical shifts were measured from the internal HDO lock signal.

4B (ii) Results

Separation of the C-4 histidine peaks from overlapping resonances is described in this section.

Figure 4.1 shows spectra of the aromatic region of Worthington ribonuclease A at pH 5.96 and pH 5.35, as well as the curve obtained by subtracting one from the other. C-2 peak assignments are those of Meadows et al. (1968); C-4 peak assignments are discussed later. The difference spectrum clearly separates those peaks which are dependent on pH from those which are not. In this pH range, only the C-2 and C-4 imidazole peaks are affected. By subtracting each spectrum from several others at different pH's, the C-4 peaks were distinguished from overlapping phenylalanine and tyrosine aromatic resonances. Several subtractions were usually required for each spectrum, because of occasional cancellation of coincident peaks. For example, in Figure 4.1, the C-4 peak of His 48 at pH 5.96 has the same chemical shift as the C-4 peak of His 119 at pH 5.35, and so these two peaks are absent in the difference spectrum. In order to reveal these peaks, subtractions from spectra at other pH's were necessary.

Figures 4.2 and 4.3 are plots of chemical shift against pH for the C-2 and C-4 peaks of Worthington ribonuclease A at low ionic strength. From each continuous curve, the apparent pK was taken as the pH of the mid-point of the main transition. The C-4 curves

FIGURE 4.1

100 MHZ SPECTRA OF RIBONUCLEASE

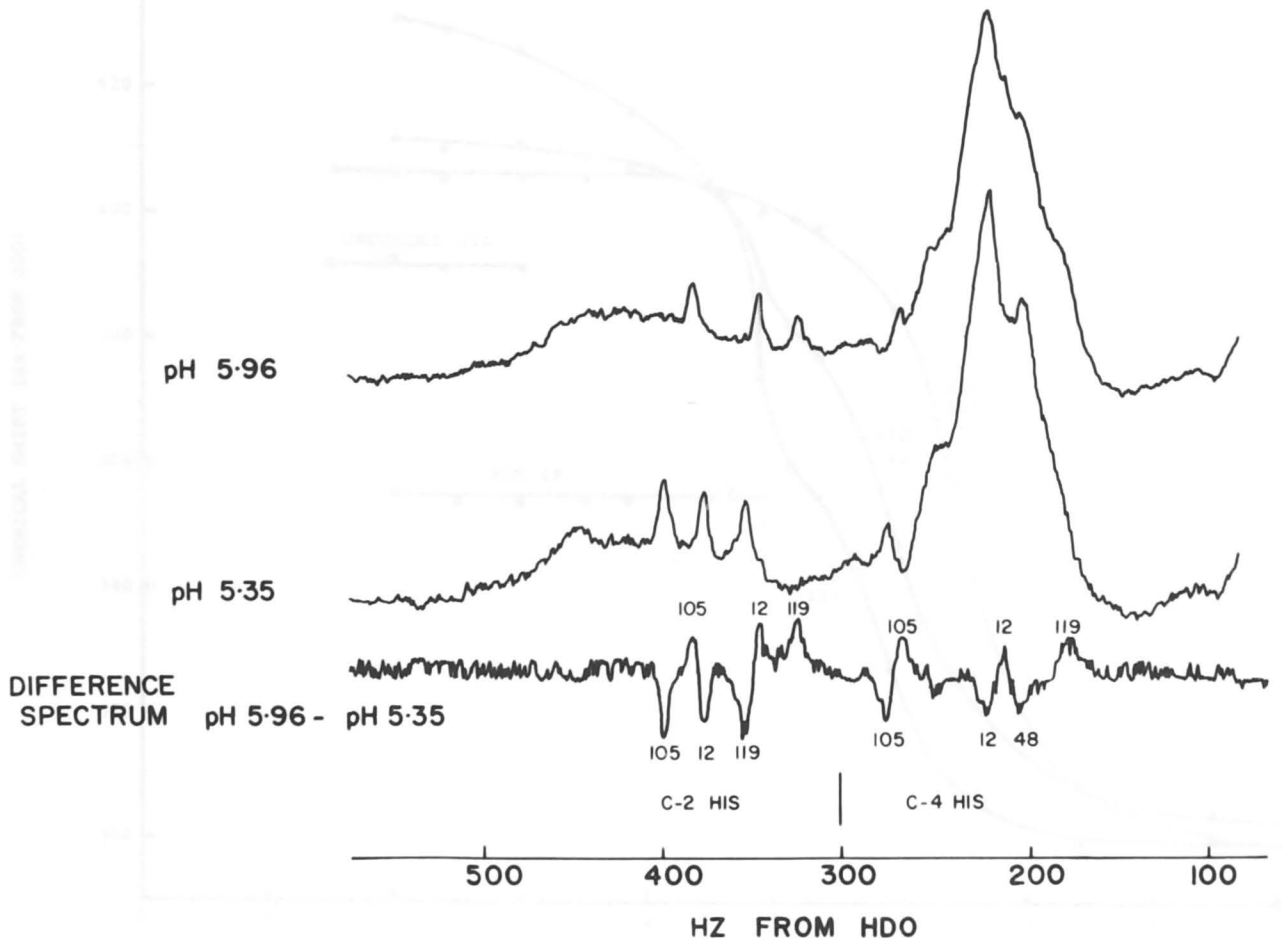


FIGURE 4.2

C-2 HISTIDINE PEAKS OF WORTHINGTON RIBONUCLEASE

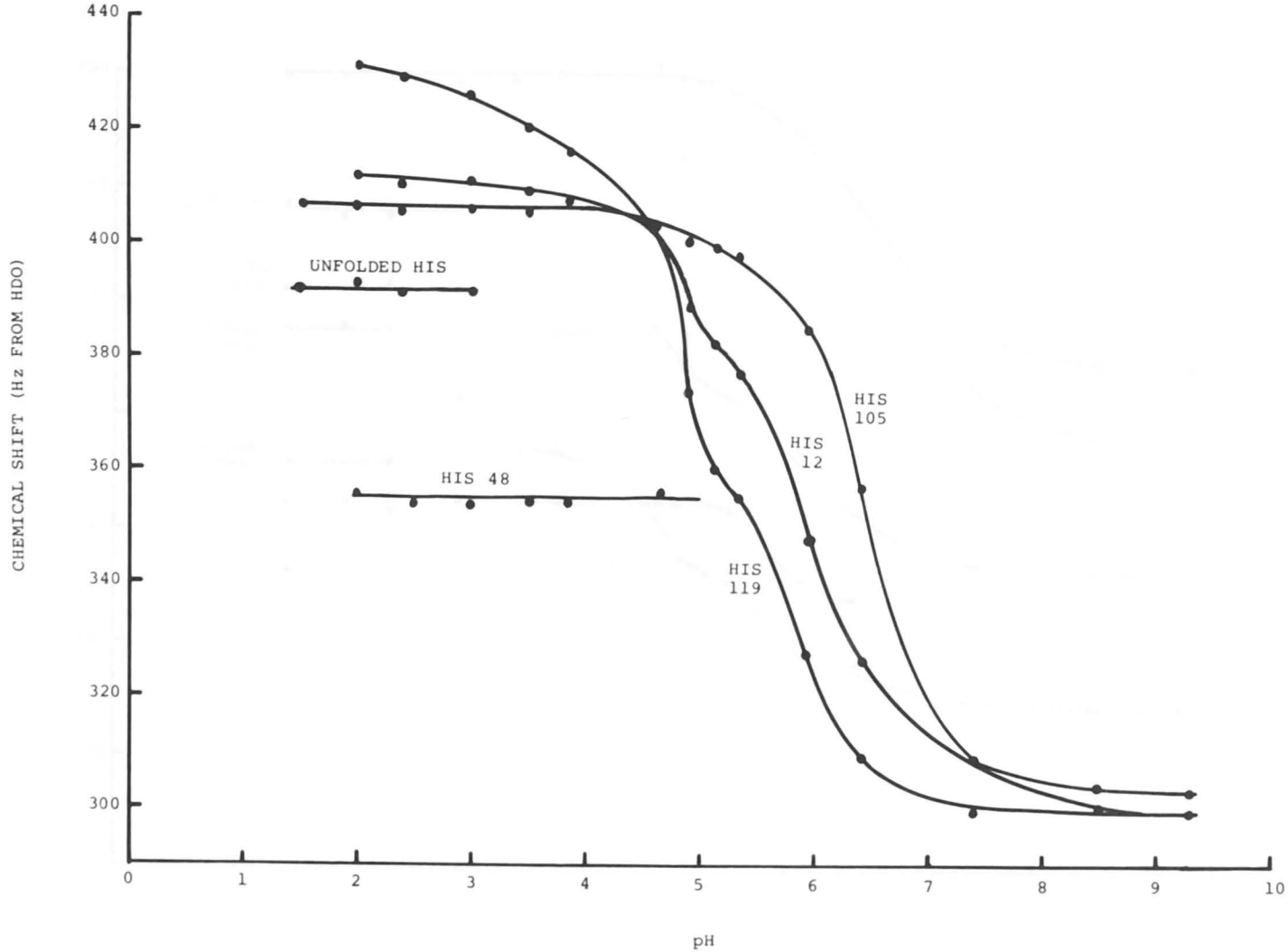
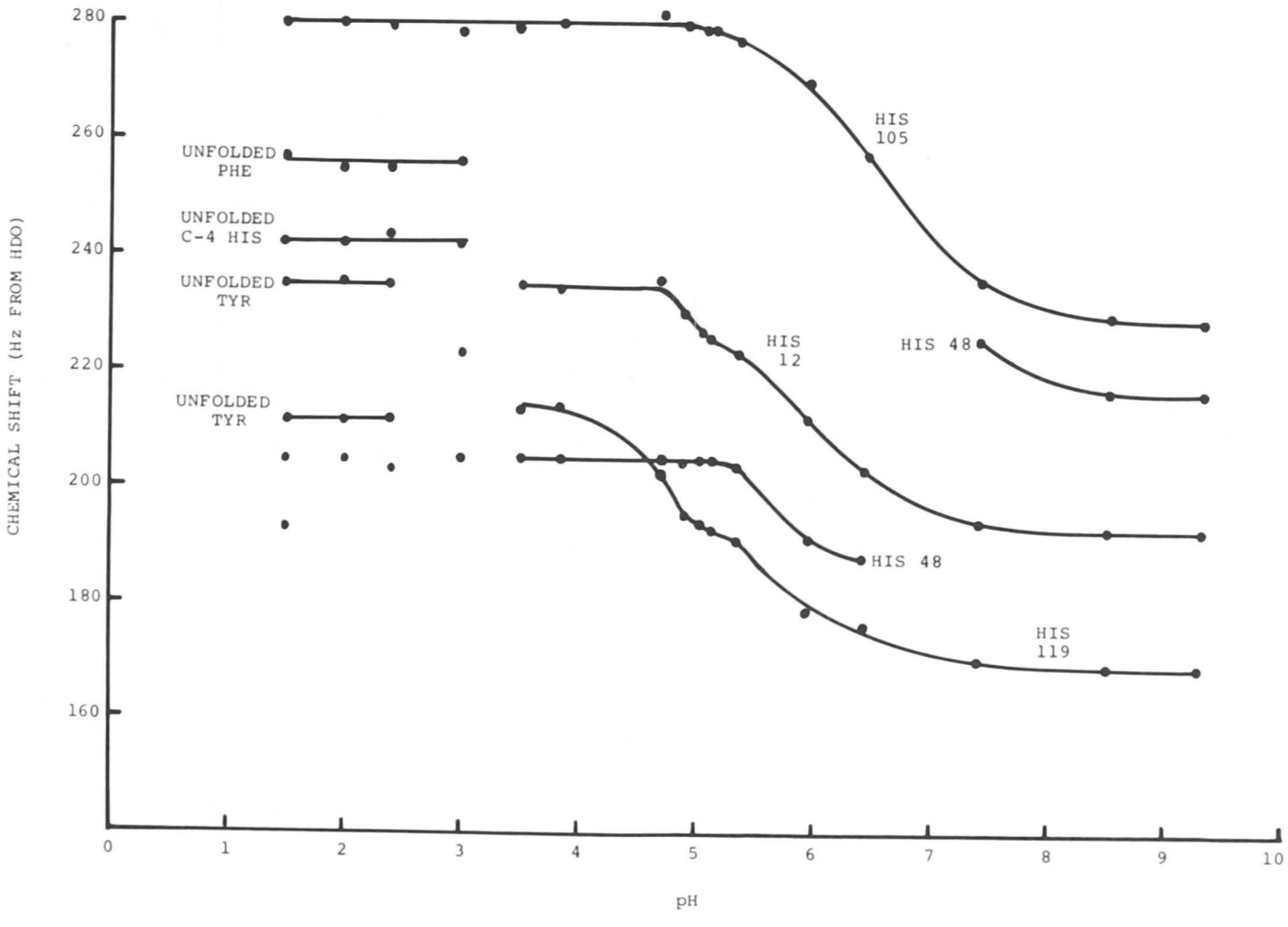


FIGURE 4.3

C-4 HISTIDINE PEAKS OF WORTHINGTON RIBONUCLEASE



reflect the same imidazole titrations as the C-2 curves, and yield the same apparent pK's, within experimental error. Each C-4 curve was therefore assigned to the same histidine residue as the corresponding C-2 curve. No pK of His 48 was obtained, since in dilute sodium chloride solutions, the C-2 peak of this residue is not visible above pH 6 (Roberts et al., 1969a; Ruterjans & Witzel, 1969). Although the C-4 peak is visible throughout the titration range, there is an apparent discontinuity in the curve (Figure 4.3).

At pH <3, acid-denaturation of ribonuclease A begins, and additional peaks corresponding to the unfolded conformations appear. The chemical shifts of these peaks are indicated in Figures 4.2 and 4.3.

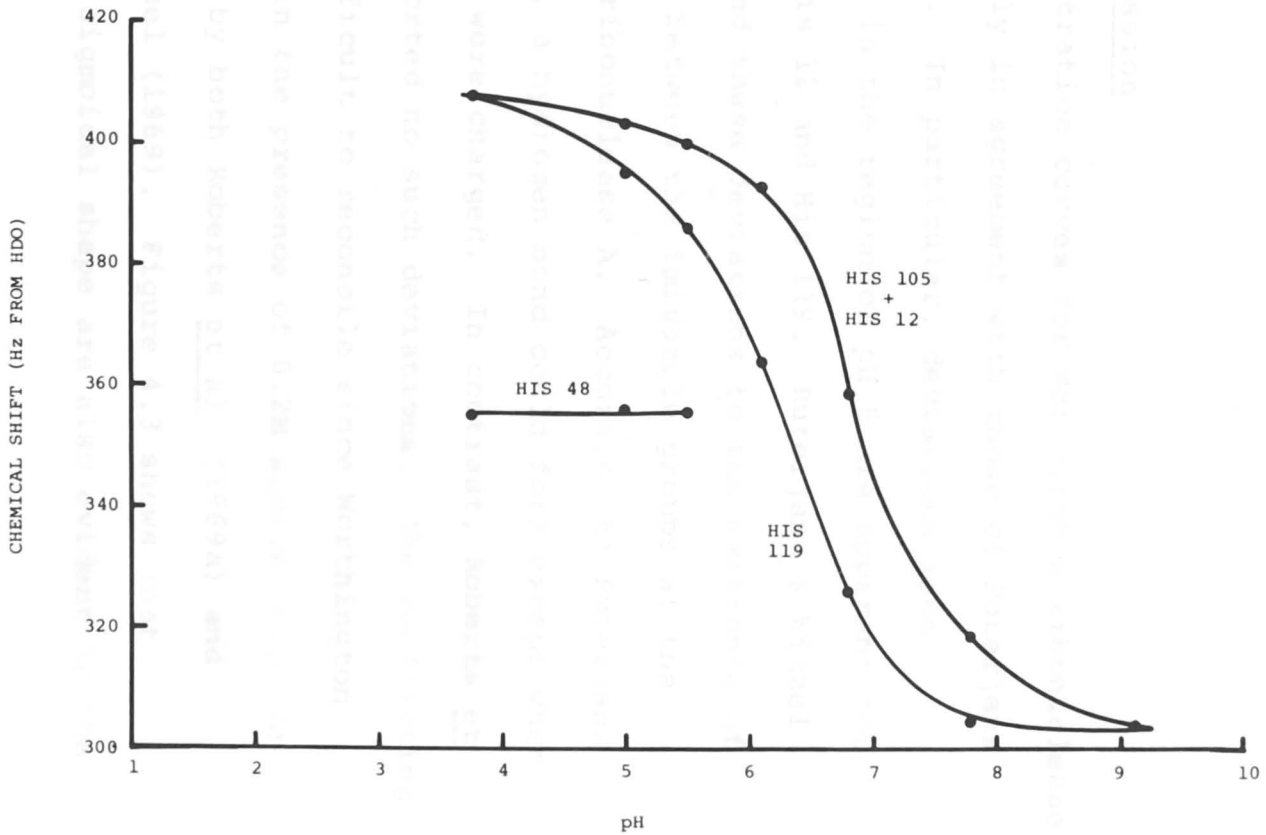
TABLE 4.1

IMIDAZOLE pK's FOR RIBONUCLEASE

Sample	Source of pK's	Histidine Residue		
		12	105	119
Worthington	Fig. 4.2	5.8	6.4	5.2
Worthington	Fig. 4.3	5.9	6.5	5.2
Sigma	Fig. 4.4	6.9	6.9	6.2

FIGURE 4.4

C-2 HISTIDINE PEAKS OF SIGMA RIBONUCLEASE



pK's obtained from the C-2 and C-4 plots of Worthington ribonuclease, as well as those from a C-2 plot of Sigma ribonuclease (Figure 4.4) are listed in Table 4.1.

4B (iii) Discussion

The C-2 titration curves for Worthington ribonuclease A are essentially in agreement with those of Ruterjans & Witzel (1969). In particular, deviations from sigmoidal shape in the region of pH 5 are apparent for the curves of His 12 and His 119. Ruterjans & Witzel (1969) attributed these deviations to the existence of a hydrogen bond between the imidazole groups at the active site of ribonuclease A. According to Ruterjans & Witzel (1969), a hydrogen bond could form except when both imidazoles were charged. In contrast, Roberts et al. (1969a) reported no such deviations. The conflicting results are difficult to reconcile since Worthington ribonuclease A in the presence of 0.2M sodium chloride in D_2O was used by both Roberts et al. (1969a) and Ruterjans & Witzel (1969). Figure 4.3 shows that deviations from sigmoidal shape are also evident in the C-4 curves.

However, no deviations are apparent in the plots of chemical shift against pH for the C-2 histidine peaks of Sigma ribonuclease (Figure 4.4). In ribonuclease S which had been crystallised from a sulphate solution (Wyckoff et al., 1967) X-ray diffraction studies showed that a sulphate anion was situated between the two charged imidazole groups at the active site (Wyckoff - personal communication). This precludes the existence of a hydrogen bond in the presence of bound sulphate.

As shown in Table 4.1, the pK's obtained for Sigma ribonuclease solutions, which contained 0.03M sulphate (section 2A), are all higher than those for Worthington ribonuclease A. Similarly, Meadows et al. (1969) obtained higher pK's for Worthington ribonuclease A in the presence of 0.08M sulphate.

As shown in Figure 4.2, the peak corresponding to the histidine residue with lowest pK moves even further downfield as the pH is reduced below 4. Such behaviour suggests the proximity of a carboxyl group, the titration of which is being reflected in the curve for the C-2 histidine peak. In the X-ray crystallographic model of Kartha et al. (1967), Asp 121 is close to His 119 but more distant from His 12. This observation provides

supporting evidence for the assignment of His 119 to the curve with lowest pK (Meadows et al., 1968 & 1969).

The apparent discontinuity in the C-4 His 48 curve is indicative of a conformational change and is consistent with the suggestion of Ruterjans & Witzel (1969) that His 48 interacts with Asp 14 at low pH, but with Tyr 25 at high pH. Nevertheless, other possible conformational changes are not excluded.

At pH <3, the appearance of peaks in the positions for unfolded aromatic residues is indicated in Figures 4.2 and 4.3. However, other peaks, not corresponding to either native or completely unfolded ribonuclease A, also appear. Their chemical shifts are represented by points lying off the curves in Fig. 4.3. The peak situated 205 Hz from HDO at pH <3 was too large to be attributed solely to the C-4 proton of His 48. These additional peaks must correspond to intermediate stages in the acid-denaturation of ribonuclease A. Their appearance impaired observations on the C-4 peaks at low pH's, and prevented extension of the C-4 curves for His 12 and 119 to pH's less than 3.5.

4C Widths of Histidine Peaks

4C (i) Proximity Broadening

Table 4.2 lists correlation times, calculated from equation (3), for a number of globular proteins. For the purpose of these calculations, all molecules were treated as spheres.

TABLE 4.2

CORRELATION TIMES FOR PROTEINS

Protein	Reference for Dimensions	Approximate "radius" (°A)	$\tau_c \times 10^9$ (sec)
Ribonuclease A	Kartha et al. (1967)	15	2.9
Lysozyme	Blake et al. (1965)	17	4.0
α-Chymotrypsin	Matthews et al. (1967)	20	6.9

For two protons on a methyl group held rigidly in ribonuclease, b = 1.8 °A, and substitution in equation (1) gives W_d = 10 Hz for a 100 MHz spectrometer. This agrees with the general observation of McDonald & Phillips (1969), that peak widths for small native proteins are controlled by over-all molecular rotation, and are typically 10-20 Hz.

However, with regard to a histidine residue in D_2O, the protons on the imidazole nitrogens are replaced by deuterons, provided there is free access to the solvent. In this case, the nearest proton to the C-2 is the C-4 proton, 4.25 °A away. Because of this large inter-proton distance, W_d for α-chymotrypsin is <0.2 Hz, which is less than experimental error. Consequently, dipole-dipole interactions between the C-2 and C-4 protons do not make a significant contribution to peak widths for the proteins in Table 4.2. For this reason, imidazole peaks are frequently only slightly broader in protein NMR spectra, than in spectra of the free amino acids. This is illustrated in Table 4.3, which shows that the C-2 peak widths for the single imidazole in native lysozyme, unfolded lysozyme, and histidine, are all equal within experimental error. In the spectrum of the free amino acid, the C-4 proton gives rise to spin-spin splitting in the C-2 peak which is a doublet with a coupling constant of 1.4 Hz (Bak et al., 1968).

The general agreement among the widths in Table 4.3 suggests that other mechanisms for broadening (e.g. spin-rotation) do not contribute significantly. Allowing a maximum value of 0.3 Hz for W_{sr}, substitution of parameters from Table 4.2 into equation (7) yields a

value for c of <1 KHz. This value agrees with the spin-rotation interaction constant for benzene protons (Pritchard & Richards, 1966).

TABLE 4.3

PEAK WIDTHS FOR IMIDAZOLE C-2 PROTONS AT 60 MHZ

Compound	Solvent	Width at Half-height (Hz)
Histidine	D_2O	2.7* (±0.3)
Lysozyme	D_2O (pH 2.8)	3.5 (±0.5)
Lysozyme	8M urea (pH 2.8) D_2O	3.0 (±0.5)

* Sweep rate : 0.3 Hz per sec.

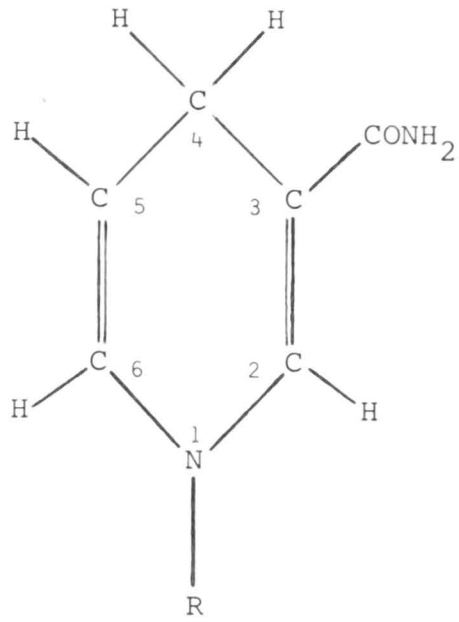
In H_2O, the lifetime of a proton on an imidazole nitrogen atom exposed to the solvent, is usually less than the correlation time for the protein molecule. This follows from the results of Ralph & Grunwald (1969) who obtained a rate constant of 1.8×10^9 sec^{-1} for the exchange of imidazole protons which are hydrogen bonded to solvent water molecules. Consequently, no additional broadening of a C-2 or C-4 peak is produced by dipolar interactions with the rapidly exchanging nitrogen protons. However, if the rate of exchange were reduced,

as for a histidyl side-chain inaccessible to the solvent, additional broadening would result.

In view of the above, no significant broadening would result from restricted mobility of accessible histidine side-chains in D_2O, at least for non-associated proteins of molecular weight up to ca 50,000. However, if a histidine side-chain is held close to another residue in the three-dimensional structure of a protein, an adjacent proton could be closer than 4.25 °A to the C-2 histidine proton. W_d would then be increased. For two hydrogen atoms which are bonded neither to each other nor to a common third atom, the minimum contact distance is 1.9 °A, according to Ramakrishnan & Ramachandran (1965). Should another proton be situated at this distance from a C-2 proton held rigidly in any of the proteins listed in Table 4.2, the peak width would be >12 Hz in 60 MHz spectra. Such a peak would be difficult to distinguish from the baseline.

Close proximity between protons may be responsible for several anomalous peak widths reported in the literature. For example, Hollis (1967) reported that the N-C-2 peak of reduced nicotinamide adenine dinucleotide (NADH) was not broadened on binding to yeast alcohol dehydrogenase, but that the N-C-4 peak

NADH



was strongly affected. Such a result is compatible with equation (1), in view of the remoteness of the N-C-2 proton from other protons, and the proximity of the N-C-4 protons to each other.

Further examples will be mentioned in subsequent sections.

4C (ii) Ribonuclease

Although the coordinates of ribonuclease A, determined by X-ray diffraction (Kartha et al., 1967), have not been published as yet, the coordinates of all

atoms (except hydrogens) in ribonuclease S were kindly supplied by Professor F.M. Richards. The conformation of the backbone is similar to that for ribonuclease A, but there are significant differences in some regions of the three-dimensional structure e.g. near His 48 (Wyckoff et al., 1967). This difference is reflected in the chemical shift for the C-2 peak of His 48, which is 20-30 Hz further downfield than in ribonuclease A (Meadows et al., 1968).

From the known configurations of amino-acid side-chains (Scheraga, 1968), the coordinates of relevant hydrogen atoms were calculated from the coordinates supplied by Professor Richards. Although the plane of the imidazole ring was defined for each histidine residue, the orientation of the ring about the β-bond was not given by Wyckoff et al. (1967). However, the orientations have since been determined (Wyckoff, 1970 - personal communication). Distances between imidazole protons and neighbouring protons were calculated for each ring. The minimum distance separating a C-2 proton from another proton was found to be 2.9 °A. This is the distance between the C-2 proton of His 48 and one of the protons on the β-carbon of Thr 82. For 100 MHz spectra of ribonuclease,

equation (1) shows that this inter-proton distance would contribute less than one Hz to the peak width. Other relevant inter-proton distances are considerably larger. Thus the X-ray data for ribonuclease S are entirely compatible with the normal widths of 3-5 Hz for the C-2 histidine peaks in spectra of ribonuclease S, published by Meadows et al. (1968).

The orientation of His 48 given by Wyckoff (1970 - personal communication) requires the C-4 proton to be ca 1.9 °A from the α-carbon proton of Ile 81. Equation (1) would then predict a contribution of 10 Hz to the width in 60 MHz spectra. Unfortunately, this and other C-4 peaks were not included by Meadows et al. (1968) in their titration studies of ribonuclease S.

Above pH 6 in chloride solutions, the C-2 proton of His 48 does not give rise to a visible peak in spectra of ribonuclease A, and this has been attributed to exchange-broadening (Roberts et al., 1969a; Ruterjans & Witzel, 1969). However, any exchange of an imidazole group between two different environments should have the same rate for both the C-2 and C-4 protons. Neither peak should therefore be selectively broadened, unless one undergoes a much larger chemical shift than the other (equation (6)). A more likely explanation is that

the C-2 proton of His 48 comes into close proximity with one or more other protons in the conformation adopted at high pH. For ribonuclease A at high pH, this explanation would require the existence of a different conformation in chloride than in acetate, since the C-2 His 48 peak is visible throughout the titration range in acetate (Roberts et al., 1969a).

Ruterjans et al. (1969) and Meadows et al. (1969) reported a variety of widths for the C-2 peaks of the active-site histidines in ribonuclease when inhibitors were added. According to the authors, these different widths were all attributable to exchange of the imidazoles between free and bound forms. However, proximity of protons may have contributed to the histidine peak widths in some cases.

4C (iii) Lysozyme

Lysozyme coordinates were kindly supplied by Dr. D.C. Phillips, and the coordinates of hydrogen atoms were calculated as for ribonuclease S. The single imidazole (His 15) is situated on the surface of the molecule, where it is hydrogen bonded to the hydroxyl group of Thr 89 (Browne et al., 1969).

The protons nearest the C-2 proton are two situated on the γ-carbon of Ile 88, and one on the β-carbon of

Arg 14. Each of these protons is ca 4.0 °A from the C-2 proton, and their total contribution to the width would be ca 0.5 Hz. Consequently, the width of the C-2 peak in spectra of native lysozyme is not significantly larger than in the spectrum of the free amino acid (Table 4.3).

The proton nearest the C-4 proton is situated on the β-carbon of His 15, and is 2.8 °A away. The dipole-dipole interaction between these two protons would contribute only 1.2 Hz to the peak width. Consequently, Meadows et al. (1967) were able to follow the pH dependence of the chemical shift of the C-4 peak, which, in spectra of native lysozyme, is superimposed on the aromatic envelope.

4C (iv) Chymotrypsin

The coordinates for all atoms (except hydrogens) in crystals of tosyl-α-chymotrypsin have recently been published by Birktoft et al. (1969). A revised set of coordinates, differing only slightly from the published ones, was kindly supplied to Dr. J.H. Bradbury by Dr. J.J. Birktoft. The coordinates of relevant hydrogen atoms were calculated from these revised coordinates, using the dimensions of amino-acid side-chains given by

Scheraga (1968). Substitution of the derived inter-proton distances in equation (1) yielded values for the peak widths of His 40 and His 57, as listed in Table 4.4. For these peaks, widths were taken as (ΣW_d + 3), since the width of each C-2 and C-4 peak in the free amino acid is 3 Hz (Table 4.3).

The predicted widths in Table 4.4 are relevant to the situation where the protons bonded to imidazole nitrogens have been exchanged for deuterons. If exchange is prevented, the width of each C-2 peak would be increased by ca 6 Hz, and each C-4 peak by ca 3 Hz. Furthermore, the C-2 peak of His 40 would be further broadened if the protons of a nearby water molecule are not exchanged.

Any imidazole peak broader than 15 Hz may be regarded as invisible under the present experimental conditions. The predicted widths in Table 4.4 suggest that the C-2 peak of His 57 would not be visible, owing to the proximity of one of the protons on the β-carbon of Ser 195. This holds despite the fact that only the upper limit for the distance (and the lower limit for the width) would be acceptable since the contact distance is not less than 1.9 °A (Ramakrishnan & Ramachandran, 1965).

TABLE 4.4

CALCULATED HISTIDINE PEAK WIDTHS FOR α-CHYMOTRYPSIN IN D_2O AT 60 MHZ[1]

Proton	Neighbouring Protons[2]	Distance (°A)	Width (Hz) $\Sigma W_d + 3$
His 40 C-2	Ser 32 CA	2.6 (±0.3)[3]	7 (5-10)[3]
	Try 141 CE3	2.9 (±0.3)	
His 40 C-4	His 40 CB	2.9 (±0.3)	5 (4-6)
	Phe 41 CA	3.6 (±0.3)	
His 57 C-2	Ser 195 CB	1.7 (±0.3)	33 (15-100)
His 57 C-4	His 57 CB	3.2 (±0.3)	4 (3-4)

[1]For approximate widths at 100 MHz, multiply by 0.9. Equation (1) shows that widths are dependent on frequency.

[2]Atoms to which these protons are bonded, have been named as in Birktoft et al. (1969).

[3]Numbers in brackets represent estimated errors in the distance, as well as the consequent range in values of the widths.

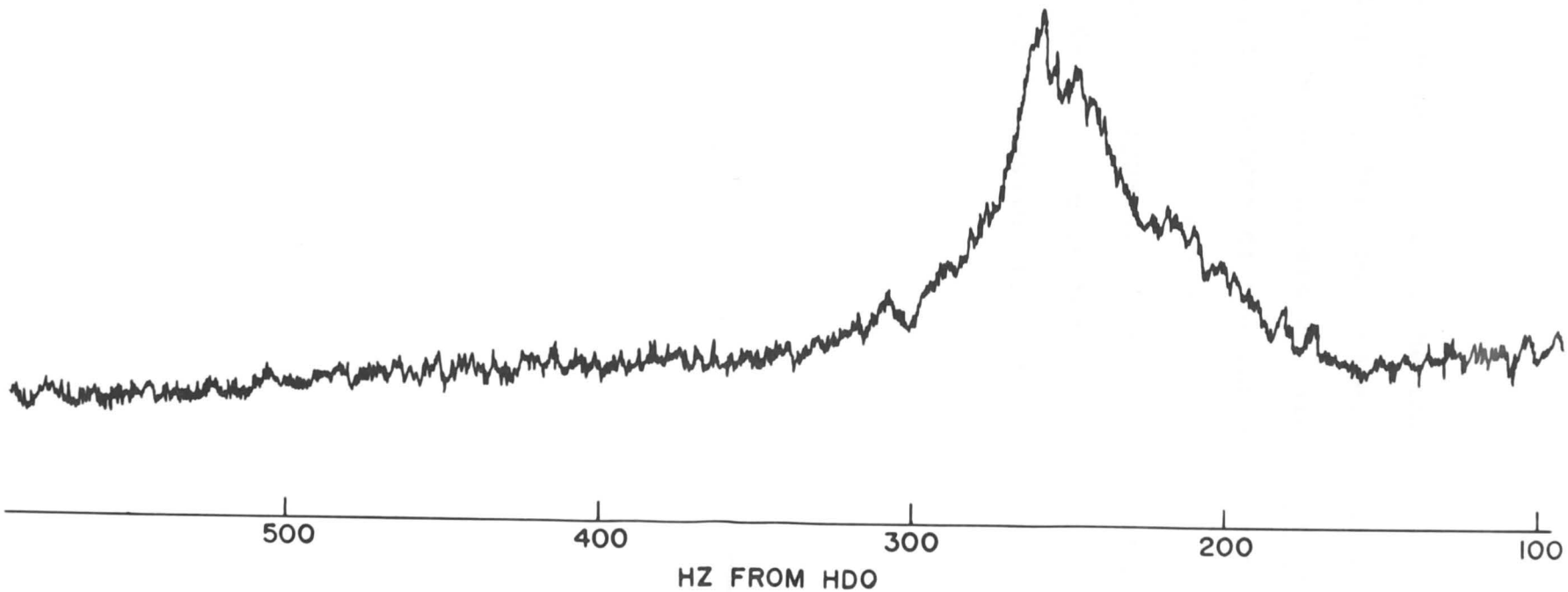
The C-2 peak of His 40 would be visible if nearby protons are exchangeable. Both C-4 peaks should be observable, although they may be broadened if neighbouring protons are not exchangeable.

Bradbury & Wilairat (1967) could not detect any histidine peak in spectra of α-chymotrypsin in D_2O. As suggested by the authors, this was partly due to association, which occurs in the absence of salt (Steiner, 1954; Tinoco, 1957). Association would increase τ_c and hence broaden the peak (equation (1)).

However, in 0.1M sodium chloride, at pH 7, Krigbaum & Godwin (1968) found α-chymotrypsin to be almost entirely monomeric up to a concentration of 6.3%. Figure 4.5 shows a 100 MHz spectrum of the aromatic region of α-chymotrypsin in D_2O under these conditions. The peak situated 310 Hz from HDO may arise from an imidazole proton, but no supporting evidence is available so far for this assignment. Further NMR studies on α-chymotrypsin are being undertaken by Mr B.E. Chapman. His preliminary results give no indication of an imidazole peak at other pH's. Thus in spectra of native α-chymotrypsin not all of the expected imidazole peaks are present. Broadening may arise by other mechanisms, or alternatively, in solutions of native α-chymotrypsin,

FIGURE 4.5

100 MHZ SPECTRUM OF α-CHYMOTRYPSIN IN D_2O (pH 7·1 , 0·1 M NaCl)



neighbouring protons may be closer to the imidazole protons than is indicated by the coordinates of tosyl-α-chymotrypsin in the crystalline state. Sigler et al. (1968) mention a slight movement of the imidazole group of His 57 when the tosyl group is bound to Ser 195 at the active site of α-chymotrypsin. Moreover, Krigbaum & Godwin (1968) have X-ray evidence for a change in shape of α-chymotrypsin on passing from the solid state into solution.

CHAPTER 5

THE UNFOLDING OF RIBONUCLEASE BY UREA AND GUANIDINE HYDROCHLORIDE

5A Introduction

Previous workers have examined the denaturation of ribonuclease by urea, but there is disagreement on several aspects of the transition.

Harrington & Schellman (1956) measured changes in viscosity, optical rotation, and ultraviolet absorption spectra for ribonuclease in 8M urea. Evidence for extensive unfolding of the enzyme molecules in this denaturant was thereby obtained. On removal of the urea by dialysis, the optical rotation returned to its value for the native enzyme, suggesting that the unfolding was reversible.

Nelson & Hummel (1962) observed that the enzymatic activity of ribonuclease decreased at urea concentrations well below those at which unfolding occurred. Furthermore, the rate at which the activity decreases in 8M urea is greater than the rate of unfolding (Barnard, 1964). It appears that the initial decrease in enzymatic activity is independent of the subsequent

gross unfolding of the ribonuclease molecule in urea. To explain this, Nelson & Hummel (1962) postulated a local uncoiling of the polypeptide chain at the active site. On the other hand, Barnard (1964) concluded that one urea molecule interacts with the di-imidazole substrate binding site at low urea concentrations, and thus inhibits activity.

At temperatures below 30°C, deviations from first-order kinetics indicate the presence of intermediate states in the unfolding of ribonuclease by urea (Nelson & Hummel, 1962; Barnard, 1964). However, above this temperature, there is no direct evidence contrary to the idea of a single-step transition. Tanford (1968) favours a single-step transition for the unfolding of ribonuclease by urea, although he regards the supporting evidence as weak. Likewise, Aune et al. (1967) consider that the unfolding of ribonuclease by GuCl is a single-step process.

In order to obtain further information on the initial decrease in activity, and the number of stages involved in the transition, an NMR study of the unfolding of ribonuclease by urea and GuCl was undertaken. The spectrum of ribonuclease in 8M urea at pH 6.5 has been

previously published by Kowalsky (1962), but no details of the transition were given.

For the present experiments, pH's of less than 5 were used, since Figure 3.5 shows that ribonuclease is completely unfolded under these conditions. In addition, equilibrium is rapidly attained at low pH (Barnard, 1964).

5B Experimental

In each unfolding experiment, a 10% solution of ribonuclease was adjusted to the required pH, and weighed amounts of re-crystallised urea were added in sequence, the pH being re-adjusted, if necessary, after each addition. Between additions of urea, the spectrum was accumulated for approximately 3 hr (100 scans) on the 60 MHz spectrometer, as described in section 2C. However, in one series, each spectrum was accumulated for 1 hr on the 100 MHz spectrometer at a sweep rate of 5 Hz per second. Finally, ribonuclease was recovered from the urea solution by elution with water on a column of Sephadex G-75, followed by freeze-drying.

Molal urea concentrations were converted to molar units according to Kawahara & Tanford (1966). Peak heights and areas were corrected for dilution accompanying increases in urea concentration.

A similar procedure was followed for the unfolding by GuCl, when the 60 MHz spectrometer was used.

5C Results

Spectra of Worthington ribonuclease in D_2O, 4.7M urea and 8.5M urea at pH 4.6 are shown in Figure 5.1. Typical sharpening of peaks, due to unfolding, is evident. The transition was followed quantitatively by measuring peak heights. For a sharp peak superimposed on a broad region of the spectrum (e.g. the methionine peak), the baseline was taken as the base of the sharp peak. Consequently, measured peak heights were not affected by any changes in underlying resonances induced by the unfolding.

From equation (9), the extent of unfolding, F, was calculated at each urea concentration for the unfolded C-2 histidine, phenylalanine, methionine and methyl peaks. These values were equal, within experimental error, for all four peaks, at each urea concentration. The average values of F are plotted against urea concentration in Figure 5.3.

Transition curves for the unfolding of Sigma ribonuclease at pH 2.8 in H_2O, and pH 4.7 in D_2O, are also shown in Figure 5.3. The advantage of using H_2O is

FIGURE 5.1

60 MHZ SPECTRA OF RIBONUCLEASE

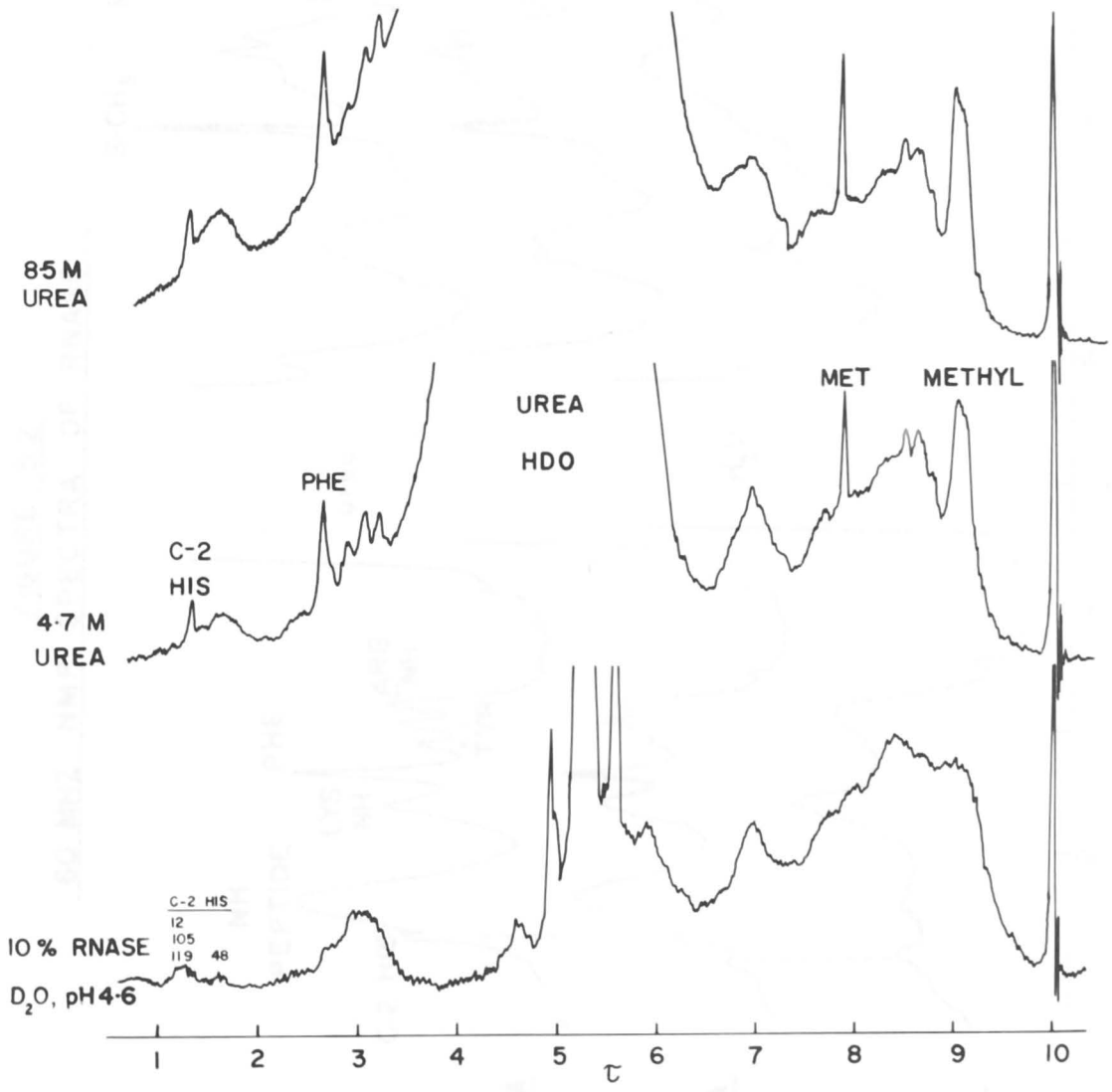


FIGURE 5.2

60 MHZ NMR SPECTRA OF RNASE

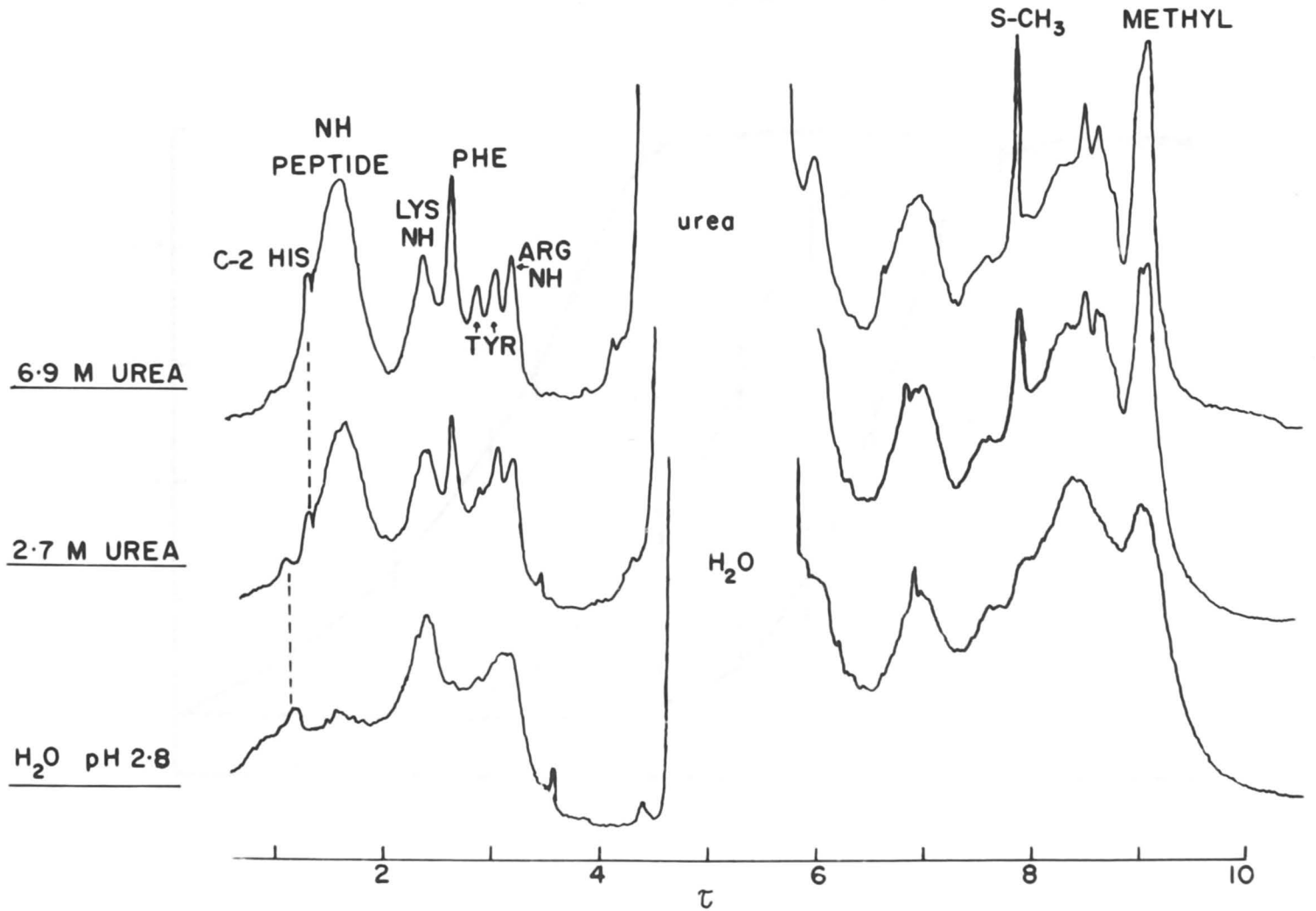
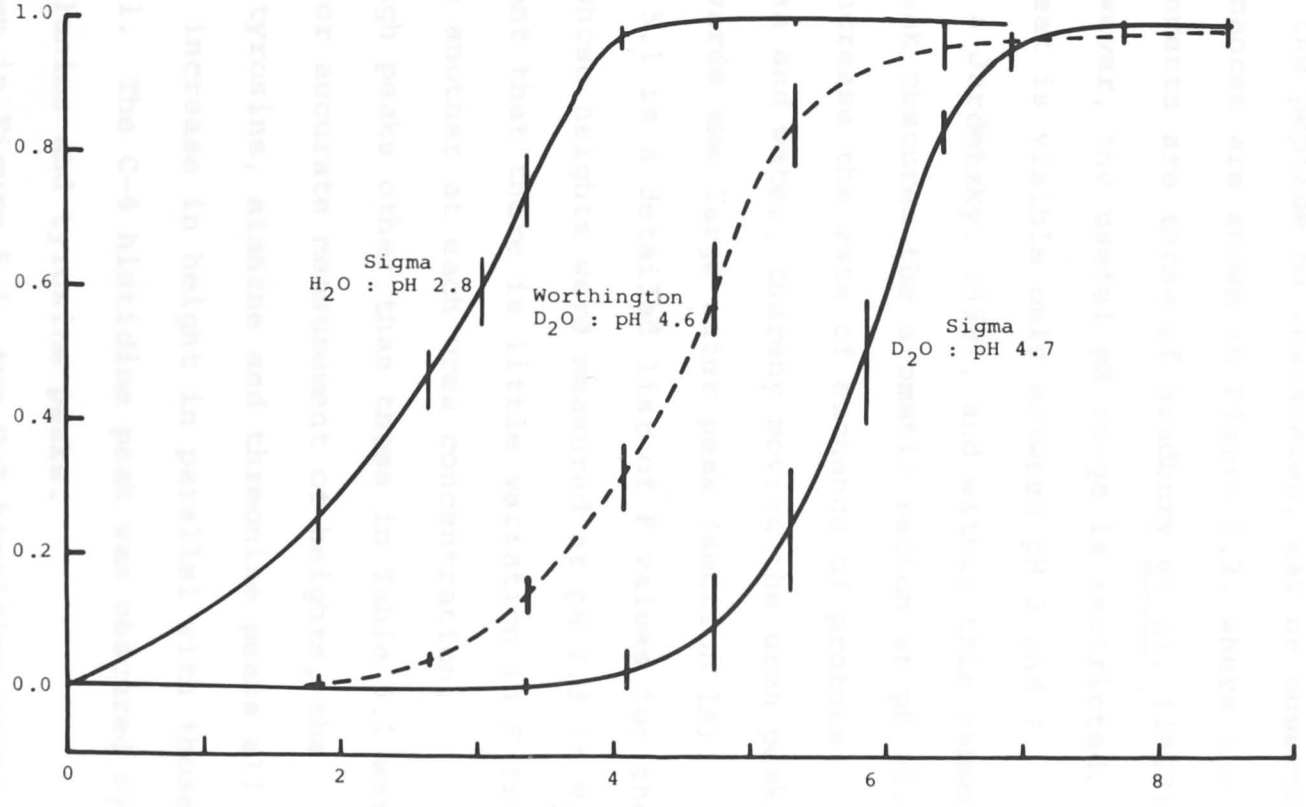


FIGURE 5.3

UNFOLDING OF RIBONUCLEASE BY UREA



Vertical lines represent standard deviations from the mean

that the NH peaks of lysine and arginine side-chains, as well as the peptide NH resonances, may be observed. These resonances are shown in Figure 5.2, where the NH peak assignments are those of Bradbury et al. (1967). In H_2O, however, the useful pH range is restricted. The arginine peak is visible only between pH 2 and 7 (Jardetzky & Jardetzky, 1958), and within this range, the urea peak obscures the aromatic region at pH >3.5. Low pH's increase the rate of exchange of protons between urea and water, thereby moving the urea peak upfield towards the large water peak (section 1A).

Table 5.1 is a detailed list of F values for the six peaks whose heights were measured at pH 2.8 in H_2O. It is evident that there is little variation in F from one peak to another at each urea concentration.

Although peaks other than those in Table 5.1 were too small for accurate measurement of heights, the lysine NH, tyrosine, alanine and threonine peaks all appeared to increase in height in parallel with those in Table 5.1. The C-4 histidine peak was obscured by the phenylalanine and tyrosine peaks.

As shown in Figure 5.1, two C-2 histidine peaks were observed at pH 4.6 in the 60 MHz spectrum of native ribonuclease. One of these is a composite peak arising

TABLE 5.1

F VALUES FOR RIBONUCLEASE IN UREA IN H_2O (pH 2.8)

Urea Molarity	F						
	Unfolded C-2 His	Peptide NH	Phe	Arginine NH	Methionine	Methyl	Mean
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.8	0.29	0.22	0.26	0.25	0.21	0.27	0.25
2.6	0.50	0.44	0.49	0.48	0.41	0.45	0.46
3.0	0.61	0.54	0.64	0.58	0.56	0.62	0.59
3.4	0.79	0.70	0.74	0.68	0.70	0.72	0.72
4.1	0.98	0.99	1.00	0.95	0.92	0.98	0.97
5.3	1.00	1.00	1.00	1.00	1.00	1.00	1.00
6.9	0.97	0.97	0.95	1.00	0.99	0.96	0.97

from His 12, 105 and 119, while the upfield peak corresponds to His 48 (Meadows et al., 1968). The C-2 peak corresponding to histidine residues in unfolded molecules is situated 0.15 ppm upfield of the native composite peak, and 0.37 ppm downfield from the native His 48 peak (Figures 4.2 and 5.1). When all molecules were unfolded, the C-2 histidine peak had the same area as the C-2 peaks in the spectrum of the native protein. However, the sum of the areas was not constant at intermediate concentrations of urea. At low urea concentrations, the C-2 histidine peaks corresponding to at least two of the residues 12, 105 and 119 decreased in area. This decrease, together with the increase in area (or height) of the unfolded C-2 peak, is shown in Figure 5.4. For comparison, the enzymatic activity curve of Barnard (1964) is also shown in Figure 5.4.

The effect of low concentrations of urea was re-examined at a pH of 5.5, where the C-2 peak of His 119 is further upfield than the C-2 peak of His 12 and 105, in the spectrum of Sigma ribonuclease. Chemical shifts of the C-2 imidazole peaks, obtained on the 100 MHz spectrometer, are plotted against urea concentration in Figure 5.5. A comparison of histidine peak areas with the area of the aromatic peak, showed that the

FIGURE 5.4

SIGMA RIBONUCLEASE (pH 4·7)

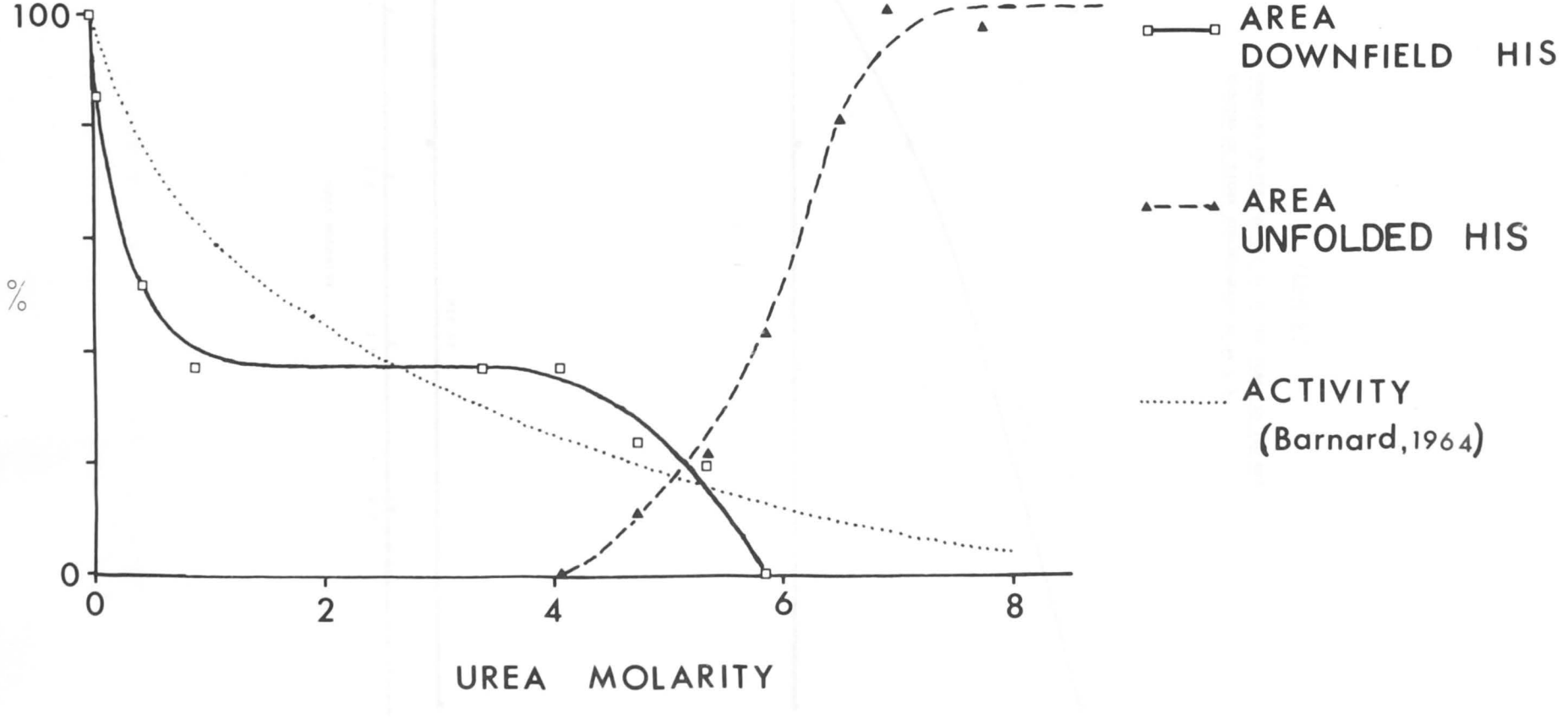
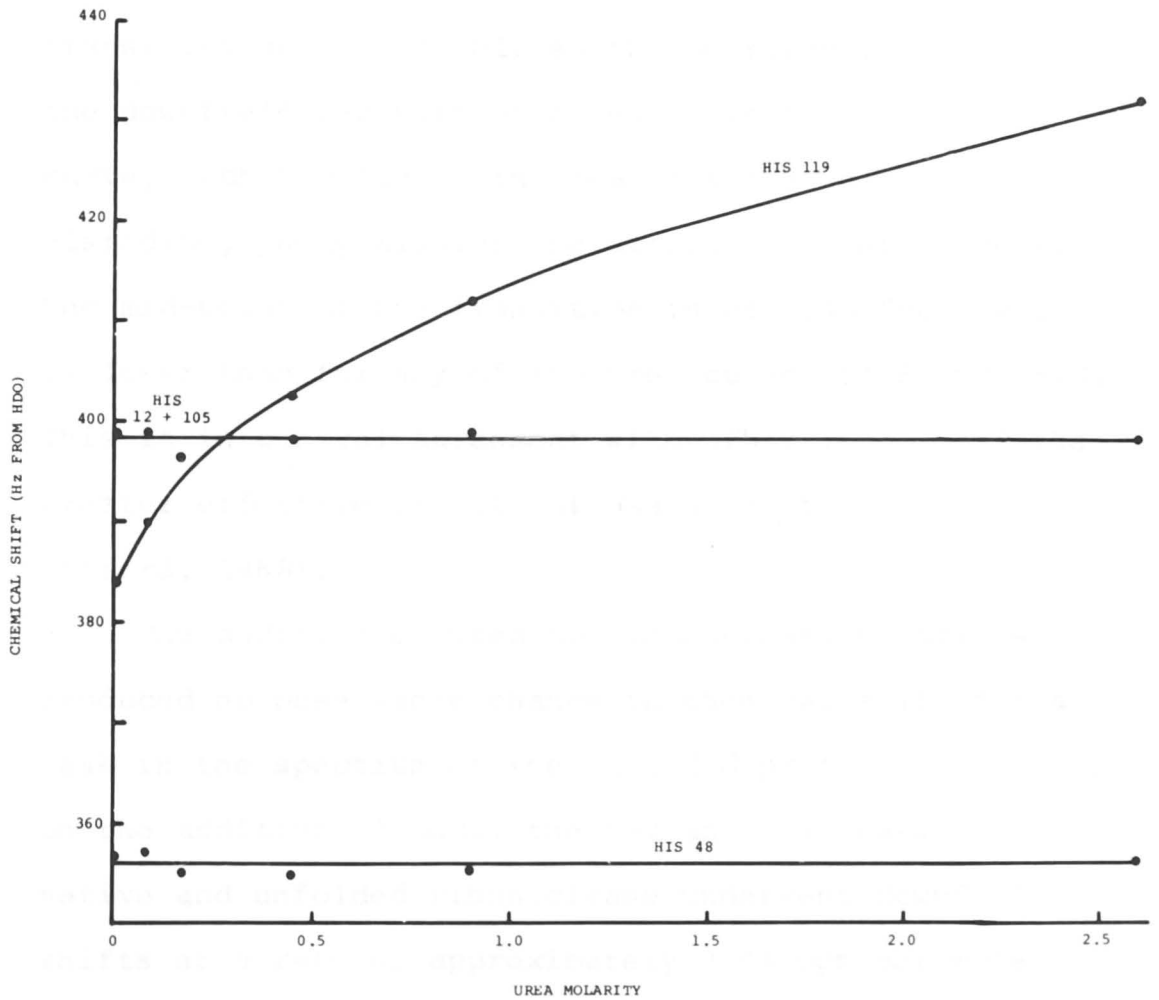


FIGURE 5.5

CHEMICAL SHIFTS FOR C-2 HISTIDINE PEAKS IN 100 MHZ SPECTRA OF SIGMA RIBONUCLEASE AT pH 5.5



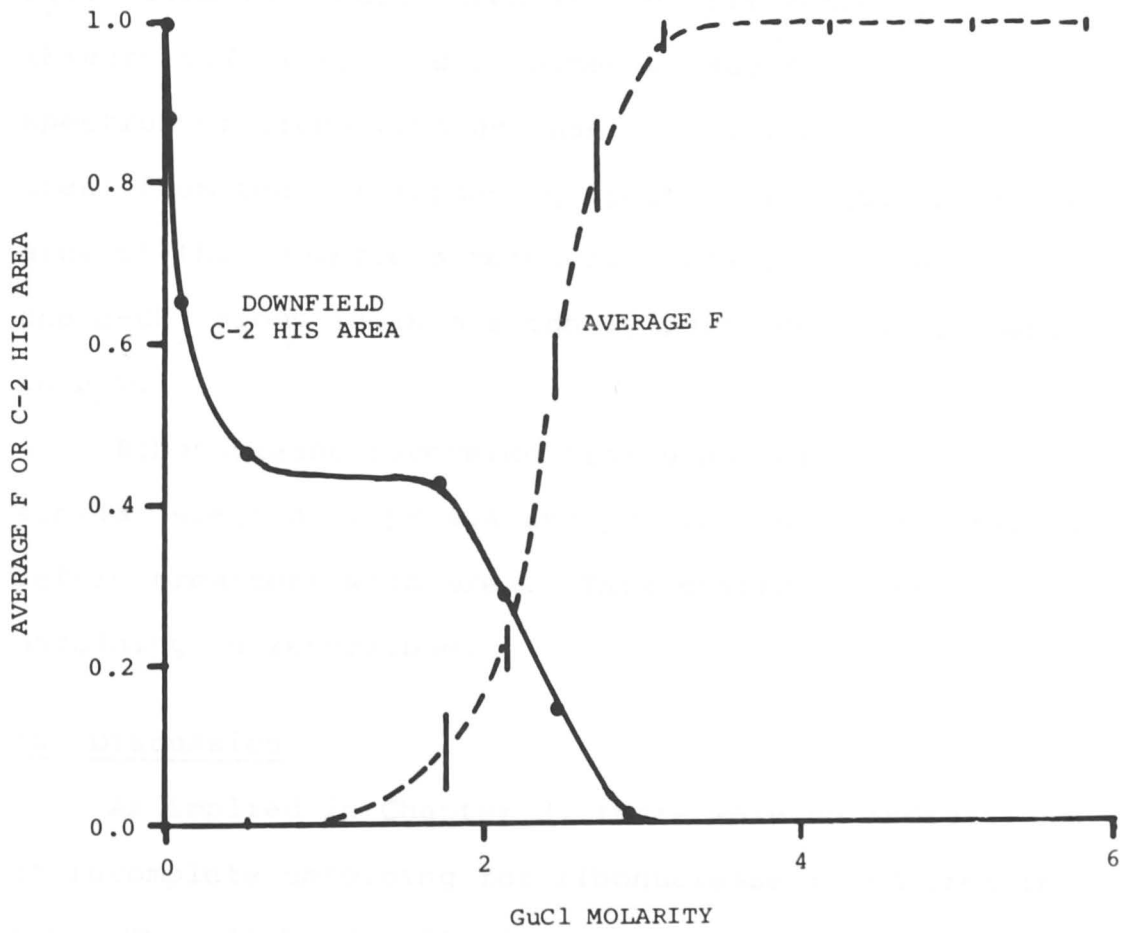
combined His 12 and 105 peak represented two protons in the absence of denaturant, but only one at 2.5M urea.

During the unfolding of Sigma ribonuclease in D_2O by GuCl at pH 4.1, all peaks appeared to increase in height in parallel, as for urea. Figure 5.6 shows the transition curve, as well as the decrease in area of the downfield C-2 histidine peak. In the transition curve, each F value is the mean for the unfolded C-2 histidine, phenylalanine, methionine and methyl peaks. The mid-point of the transition is at 2.4M GuCl, which is lower than for any of the urea curves in Figure 5.3. This is in general agreement with other reports of the greater effectiveness of GuCl as a denaturant (e.g. Tanford, 1968).

The addition of urea to ribonuclease solutions produced no observable change in chemical shift for any peak in the spectrum of the unfolded protein. However, on the addition of GuCl, the C-2 and C-4 peaks of both native and unfolded ribonuclease underwent downfield shifts at a rate of approximately 0.04 ppm per mole of added GuCl. Similarly, the methionine and methyl peaks moved downfield at a rate of 0.03 ppm per mole of GuCl, but the phenyl peak underwent a much smaller shift. These values are consistent with the general range of 5

FIGURE 5.6

UNFOLDING OF SIGMA RIBONUCLEASE BY GUANIDINE HYDROCHLORIDE (pH 4.1)



Vertical lines represent standard deviations from the mean

to 12 Hz reported by McDonald & Phillips (1969) for the downfield chemical shifts of protein peaks in 6M guanidine at 220 MHz.

At all urea concentrations where the methionine peak was visible, there was a shoulder on the downfield side of this peak for ribonuclease in H_2O, but not in D_2O. This is clearly seen in the difference spectrum (Figure 5.7) which was obtained by subtracting the spectrum of Sigma ribonuclease in D_2O (pH 2.7, 5.3M urea) from the corresponding spectrum in H_2O. From the area of this downfield methionine peak, it appears that one $S-CH_3$ group is in a slightly perturbed environment in H_2O.

Ribonuclease recovered from urea solutions had similar spectra at pH 4.4 and pH 6.0, to those obtained before treatment with urea. This confirms that the unfolding is reversible.

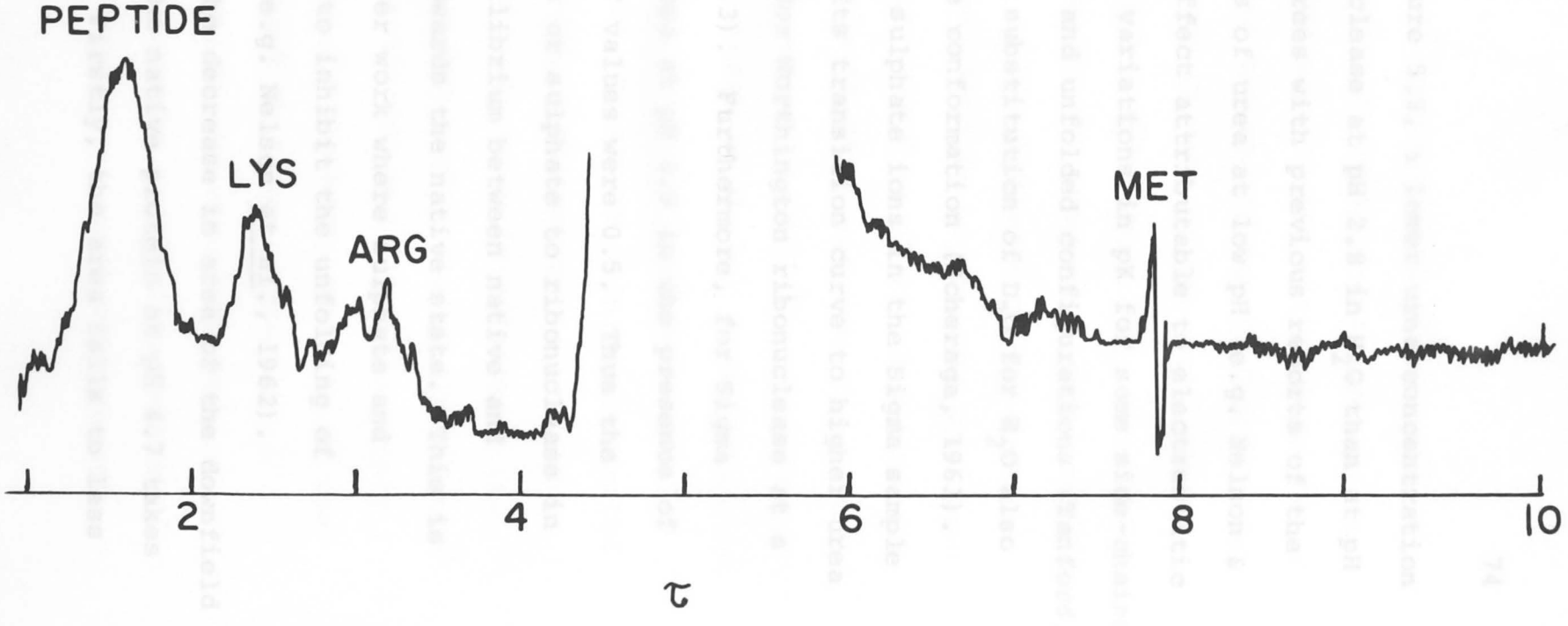
5D Discussion

As implied in Chapter 3, there were no indications of incomplete unfolding for ribonuclease in 8M urea in D_2O. The slight downfield chemical shift of a peak due to one of the methionine residues in H_2O (Figure 5.7) was the only suggestion of a residual non-covalent interaction for ribonuclease in 8M urea.

FIGURE 5.7

RIBONUCLEASE IN 5·3 M UREA pH 2·7

$H_2O - D_2O$



As shown in Figure 5.3, a lower urea concentration unfolds Sigma ribonuclease at pH 2.8 in H_2O than at pH 4.7 in D_2O. This agrees with previous reports of the greater effectiveness of urea at low pH (e.g. Nelson & Hummel, 1962) - an effect attributable to electrostatic interactions, and to variations in pK for some side-chains between their native and unfolded configurations (Tanford, 1969). However, the substitution of D_2O for H_2O also stabilises the native conformation (Scheraga, 1961).

The presence of sulphate ions in the Sigma sample (section 2A) shifts its transition curve to higher urea concentrations than for Worthington ribonuclease at a similar pH (Figure 5.3). Furthermore, for Sigma ribonuclease in 8M urea at pH 4.9 in the presence of 0.1M phosphate, all F values were 0.5. Thus the addition of phosphate or sulphate to ribonuclease in urea, shifts the equilibrium between native and unfolded molecules towards the native state. This is consistent with earlier work where sulphate and phosphate were found to inhibit the unfolding of ribonuclease by urea(e.g. Nelson et al., 1962).

In Figure 5.4, the decrease in area of the downfield histidine peaks for the native protein at pH 4.7 takes place in two stages. Firstly, the area falls to less

than 0.4 of its initial value at urea concentrations in the range where the activity rapidly decreases. The second stage, where the area of the native peaks drops to zero, corresponds to the unfolding of ribonuclease, as shown by the increase in area of the peak arising from unperturbed histidine residues.

At pH 5.5, in the 100 MHz spectra, a downfield shift of the C-2 peak of His 119 occurred at low urea concentrations (Figure 5.5). It is possible that such a shift may have also occurred at pH 4.7, but with the lower sensitivity of the 60 MHz spectrometer, no shift was apparent.

If the initial decrease in enzymic activity at low urea concentrations were due to a local uncoiling of the polypeptide chain at the active site, as suggested by Nelson & Hummel (1962), a sharpening of resonances would be observed. Instead, there is a decrease in area of the native histidine peak corresponding to one or more of the residues 12, 105 and 119, while other peaks remain unchanged. Furthermore, movement of the imidazole group of His 119 to a more perturbed environment (as indicated by the change in chemical shift) would not be expected to result from unfolding of the active site. The observed changes in the NMR

spectrum are most easily explained in terms of an interaction between urea and the active-site histidines 12 and 119, as suggested by Barnard (1964). As shown in section 4C, a decrease in mobility of the histidine side-chains could not account for the extreme broadening. It may be caused by a slight conformational change which brings other protons into closer proximity with the C-2 protons, or alternatively, it may be due to exchange of the imidazole rings between free and bound forms. Similar broadening of the C-2 peaks of His 12 and 119 in the presence of other inhibitors has been attributed to exchange by Ruterjans & Witzel (1969) and by Meadows et al. (1969). These workers also reported similar downfield chemical shifts.

Further evidence against the local uncoiling hypothesis is provided by the electron paramagnetic resonance studies of Smith (1968). For ribonuclease spin-labelled at His 12, the native spectrum was unchanged in the presence of 4M urea, but there was considerable peak-narrowing in 8M urea. This indicates that the conformation of the active site is not unfolded in 4M urea.

For ribonuclease in GuCl, a similar two-stage decrease in area of the histidine peak was observed

(Figure 5.6) and the same interpretations apply.

As stated on page 12, the presence of intermediate stages in the unfolding of proteins can sometimes be recognised by (i) the appearance of additional peaks in the spectrum, or (ii) the non-equivalence of F values in the transition region. However, neither of these criteria indicate intermediates in the unfolding of ribonuclease by urea or GuCl. Apart from the downfield composite C-2 histidine peak, and solvent peaks, each spectrum in the transition region was a linear combination of spectra corresponding to native and completely unfolded conformations. As noted above, there is the possibility of a slight conformational change at the active site at low concentrations of urea or GuCl, but there is no evidence, so far, for intermediates in the main unfolding transition. The only hint of such intermediates arises from the pronounced skewness in the transition curve for the unfolding of ribonuclease by urea at pH 2.8. The presence of asymmetry in transition curves has been interpreted by Schellman (1958) in terms of intermediate conformations in rapid equilibrium.

To summarise, there is no evidence for stable intermediates in the unfolding of ribonuclease by urea

or GuCl in the pH range 4-5 at 33.4°C, but the asymmetric urea transition curve at pH 2.8 may indicate intermediates.

The significance of F may now be considered. All peaks corresponding to unfolded molecules retained the same chemical shift throughout the transition in urea. Hence there is no rapid exchange between native and unfolded forms; rapid exchange would give rise to a common peak with a chemical shift varying as the transition progresses (equation (5)). Furthermore, there was no change in width for peaks corresponding to unfolded molecules. This shows that W_e is negligible, and that the exchange is sufficiently slow for equation (10) to be applicable. Consequently, for those pH's where the transition in urea is single-step, $F = \alpha$.

With regard to the unfolding of ribonuclease by GuCl, peaks corresponding to unfolded molecules underwent changes in chemical shift. However, these may be attributed to the effects of solvent rather than to exchange, since the changes continued at concentrations of GuCl beyond the transition region. Hence, once again $F = \alpha$.

5E Methylurea

A preliminary examination of the unfolding of ribonuclease by methylurea was undertaken with a view to following changes in the methyl peak of this denaturant, as well as changes in protein peaks. Observations on the NH peak of urea are complicated by exchange of urea protons with water protons.

For a range of concentrations of methylurea, 50 mg Sigma ribonuclease was dissolved in 0.5 ml methylurea-D_2O solution at pH 3. Before and after addition of protein, the volume was measured in a graduated NMR tube. The volume increased by 0.03 ml for each solution, when the protein sample was added.

A single scan of the spectrum of each protein solution was recorded at 100 MHz, to enable the height of the protein methyl peak to be measured. By the criteria in Chapter 3, there was no evidence for incomplete unfolding of ribonuclease in 8.5M methylurea. F values calculated from the ribonuclease methyl peak are shown in Figure 5.8.

Each methylurea solution, in the presence and absence of protein, was scanned sixteen times by the 60 MHz spectrometer. Under programme control, the height of the denaturant methyl peak was measured at the end

of every scan. At the completion of the series of sixteen scans, the average height, together with the variance, was printed by the teletype. For each concentration of methylurea, the ratio (R) of the height in the presence of ribonuclease to the height in the absence of ribonuclease is shown in Figure 5.8. The peak height in the presence of ribonuclease was corrected for the volume change.

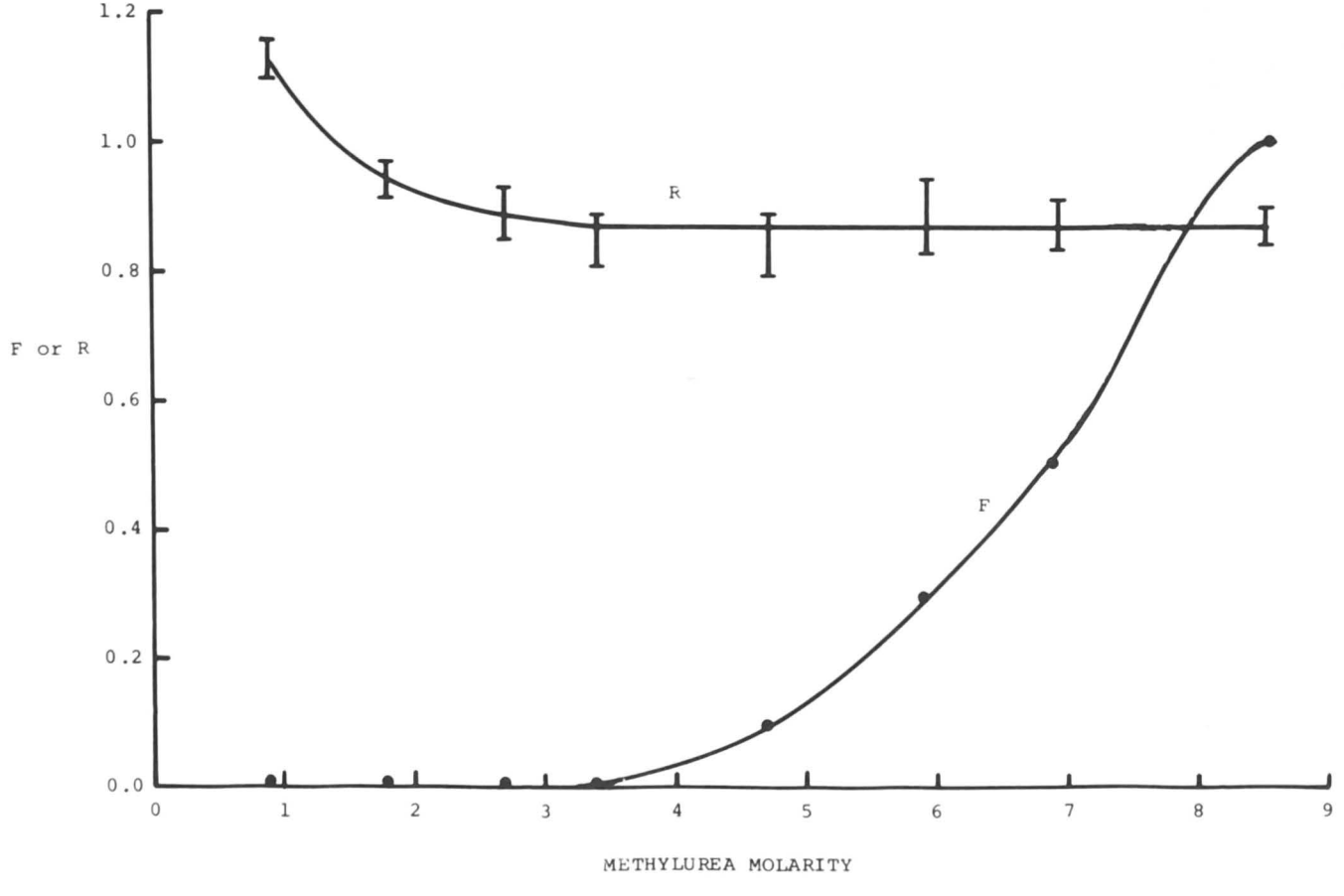
The chemical shift (7.35τ) of the denaturant methyl peak for an 8M methylurea solution was not altered significantly by the addition of ribonuclease.

Figure 5.8 shows that the mid-point of the transition is at 7M methylurea, which is considerably higher than for the unfolding by urea (Figure 5.3). This result agrees with previous reports that urea is effective at lower concentrations than methylurea (Gordon & Jencks, 1963).

The increase in R at low methylurea concentrations most likely arises from a reduction in correlation time when protein is added (equation (1)). This would correspond to less solvent structure around methylurea molecules in the presence of protein. The addition of salts to water may increase or decrease the ordering of solvent water molecules around ions, depending on the

FIGURE 5.8

RIBONUCLEASE IN METHYLUREA



Vertical lines represent standard deviations.

nature of the salt (e.g. von Hippel & Schleich, 1969). The decrease in R at higher methylurea concentrations may be a consequence of the binding of methylurea to protein molecules, as found by Gordon & Warren (1968). However, from the NMR data alone, it is difficult to distinguish between binding and changes in solvent structure.

CHAPTER 6

THE UNFOLDING OF RIBONUCLEASE BY FORMIC ACID

6A Introduction

The action of formic acid on ribonuclease has been studied previously by Josefsson (1958 & 1962). One effect observed was a loss in enzymatic activity, and this was found to be reversible, in confirmation of an earlier report by Anfinsen et al. (1954). Electrophoretic and other evidence suggested that N → O acyl shifts occurred in anhydrous formic acid (Josefsson, 1958). However, this conclusion has been refuted by Narita (1959), Smillie & Neurath (1959) and Kienhuis et al. (1959) who obtained evidence for the formylation of serine and threonine hydroxyl groups. These workers were unable to detect additional free amino groups after treatment with anhydrous formic acid. Furthermore, radioactive groups were incorporated into the covalent structure of ribonuclease after treatment with C^{14}-formic acid (Narita, 1959).

The product studied by Josefsson (1958 & 1962) was the inactivated protein obtained by precipitation with ethyl ether after treatment in anhydrous formic acid.

Unfortunately, it was not possible to decide whether the aggregation (detected by sedimentation in aqueous solution) was produced by the formic acid treatment or by the precipitation step. A direct investigation of the structure of ribonuclease, as it exists in formic acid, therefore seems warranted.

As noted in Chapter 3, ribonuclease is completely unfolded in anhydrous formic acid. In addition, formylation of the serine and threonine groups was detected by a downfield shift of the peak due to their β-carbon protons. Preliminary experiments indicated that no appreciable formylation occurred at formic concentrations of less than 60% for an immersion time of one day. Hence concentrations of less than 60% formic acid were chosen for investigations of the reversibility of the unfolding.

6B Experimental

In the unfolding experiments, small volumes of d_2-formic acid were successively added, by means of micropipettes, to a Sigma ribonuclease solution with an initial concentration of 15% w/v at pH 4.5 in 1.0 ml D_2O. A 60 MHz NMR spectrum was accumulated for 3 hr after each addition, as described in section 5B. This was continued until the acid concentration was 16% by

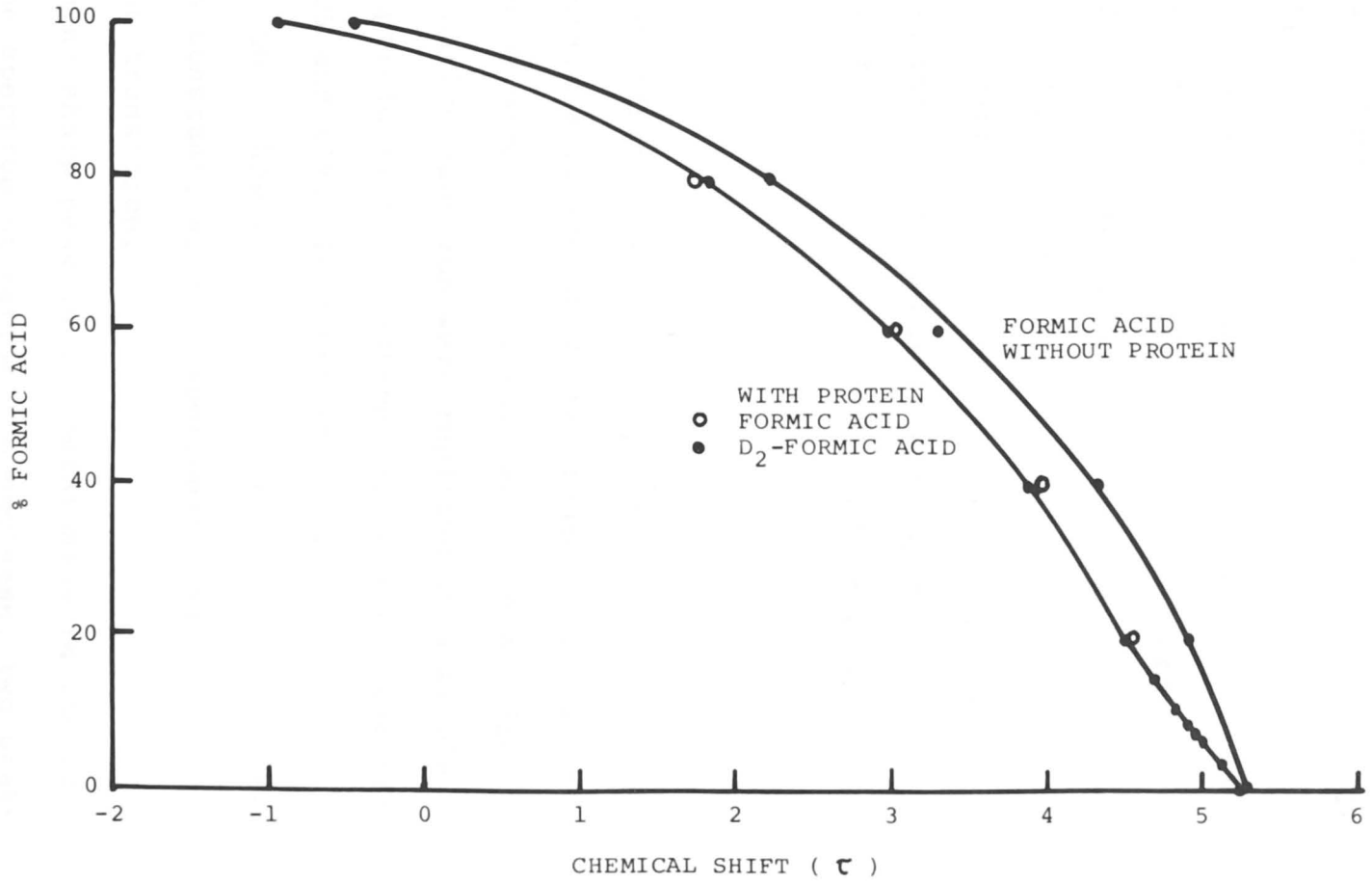
volume. For higher concentrations, protein solutions were prepared by mixing appropriate volumes of acid and D_2O. The concentration of d_2-formic acid in each solution was estimated from the height of the formic CH peak, as well as from the relative volumes of acid and D_2O. However, neither of these estimations was as reliable as a measurement of chemical shift. The HDO peak moved downfield as the concentration of d_2-formic acid was increased, owing to exchange of protons and deuterons among water and acid molecules. A calibration curve of HDO chemical shift against formic acid concentration was plotted for subsequent determination of formic concentrations in refolding experiments. This calibration curve is shown in Figure 6.1.

To investigate the refolding of ribonuclease by removal of formic acid, a 5 ml solution of protein dissolved in 30% d_2-formic acid was placed in a graduated cylinder, covered across the bottom by a sheet of dialysis tubing. The lower portion of the cylinder was immersed in 50 ml D_2O which was stirred continuously. Passage of solvent through the semi-permeable membrane was allowed to continue for one day. Initially, owing to osmotic pressure effects, the volume of liquid in the cylinder increased, thereby diluting the protein

FIGURE 6.1

FORMIC ACID CALIBRATION CURVES

COMBINED HDO & FORMIC OH PEAK



solution. At intervals, the volume change was noted, 0.5 ml protein solution was withdrawn from the cylinder, and the NMR spectrum was recorded. The concentration of d_2-formic acid in each sample was estimated from the chemical shift of the HDO peak, using the calibration curve in Figure 6.1. Protein peak heights were corrected for dilution effects.

In another experiment, ribonuclease was progressively unfolded by additions of d_2-formic acid up to a concentration of 16%, and then refolded by the above procedure.

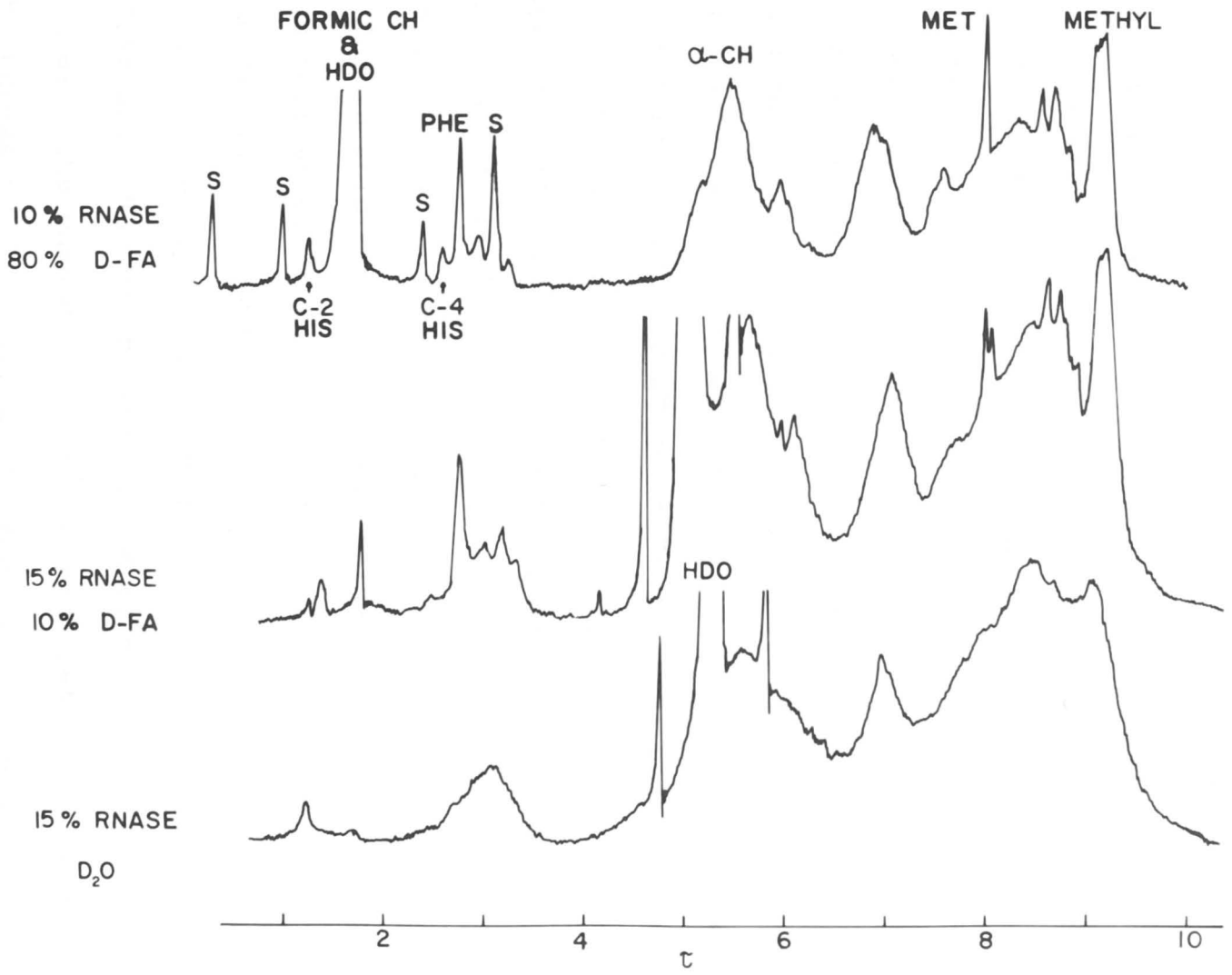
6C Results

Spectra of ribonuclease in D_2O, 10% d_2-formic acid and 80% d_2-formic acid are shown in Figure 6.2. As for the unfolding by urea and GuCl, the two C-2 histidine peaks in the native spectrum were replaced by a single peak, 0.15τ upfield from the native C-2 peak corresponding to His 12, 105 and 119. In contrast to the results with urea and GuCl, however, the sum of the areas of the C-2 peaks was constant, within experimental error, throughout the transition.

Although no sharp peak in the methionine region is visible in the spectrum of native ribonuclease, two peaks

FIGURE 6.2

60 MHZ SPECTRA OF RIBONUCLEASE



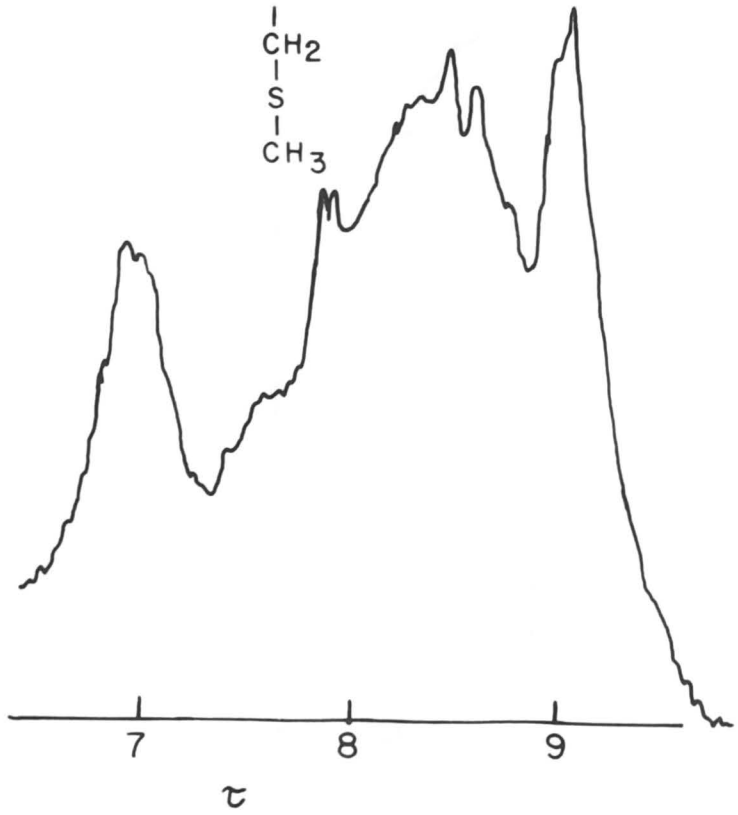
at 7.88 and 7.93τ, are present at intermediate concentrations of d_2-formic acid, while only the downfield peak is visible at formic concentrations greater than 40% (Figure 6.2). The two peaks were separated by 3Hz in 60 MHz spectra, and by 5 Hz in a 100 MHz spectrum of ribonuclease in 15% d_2-formic acid. This shows that the doublet is not due to spin-spin splitting, but represents a difference in chemical shift.

In order to check that both peaks at intermediate concentrations were due to methionine residues, 2 µl hydrogen peroxide (30%) was added to 0.5 ml ribonuclease in 10% formic acid. As shown in Figure 6.3, both peaks at 7.88 and 7.93τ disappeared and were replaced by a single peak at 7.22τ, which is the position for the $S-CH_3$ peak of methionine sulphoxide. Under more vigorous conditions (20 µl hydrogen peroxide in 0.5 ml 96% formic acid) the single methionine peak was replaced by another at 6.77τ in the position of the $S-CH_3$ peak of methionine sulphone.

The above experiment confirms that the two peaks at 7.88 and 7.93τ can be attributed to methionine residues. The change in height of each methionine peak with increasing d_2-formic acid concentration is shown in Figure 6.4. Two methionine peaks with the same

FIGURE 6.3

RIBONUCLEASE
in 10% FORMIC ACID



PARTIALLY OXIDISED
RIBONUCLEASE

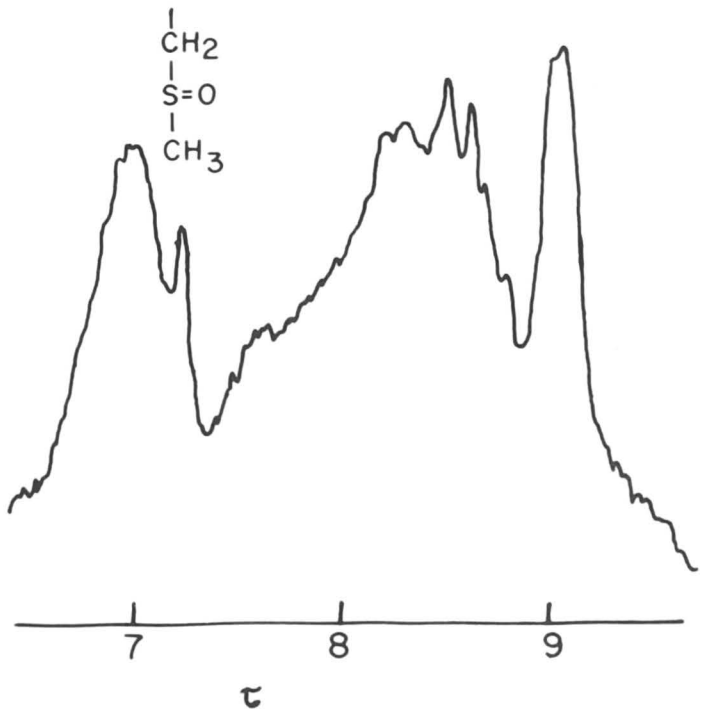
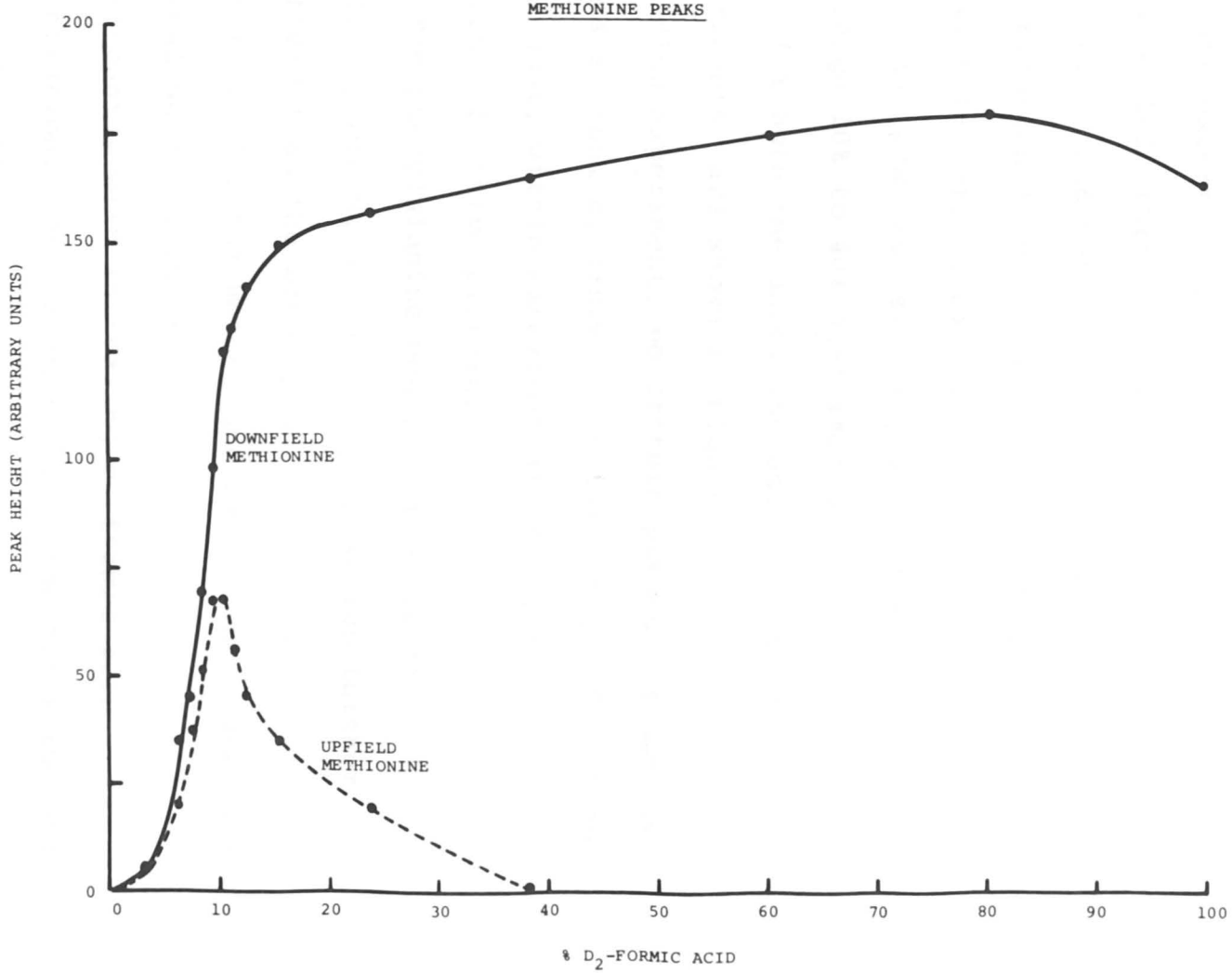


FIGURE 6.4

RIBONUCLEASE IN D_2-FORMIC ACID

METHIONINE PEAKS



chemical shifts were also observed in a spectrum of Worthington ribonuclease in 20% d_2-formic acid.

The extent of unfolding, F, was calculated for the unfolded C-2 histidine, phenylalanine, downfield methionine, and methyl peaks. At each d_2-formic acid concentration these values were equal, within experimental error, except that F for the downfield methionine peak was slightly lower than the others in the range 10% to 40% d_2-formic acid. The average values of F, for both the unfolding and the refolding experiments, are shown in Figure 6.5. During the refolding experiment, no protein passed through the dialysis tubing, since the final corrected peak heights were equal, within experimental error, to those in a spectrum of native protein.

The phenylalanine peak, in the spectrum of the unfolded protein, appeared in a position further downfield than the broad peak for the native protein. However, as for the methyl and methionine peaks, the chemical shift of the unfolded peak did not alter significantly with increasing d_2-formic acid concentration. On the other hand, the histidine and α-CH peaks all underwent downfield shifts, as shown in Figure 6.6. The C-4 histidine peak was not visible at

FIGURE 6.5

RIBONUCLEASE IN D_2-FORMIC ACID

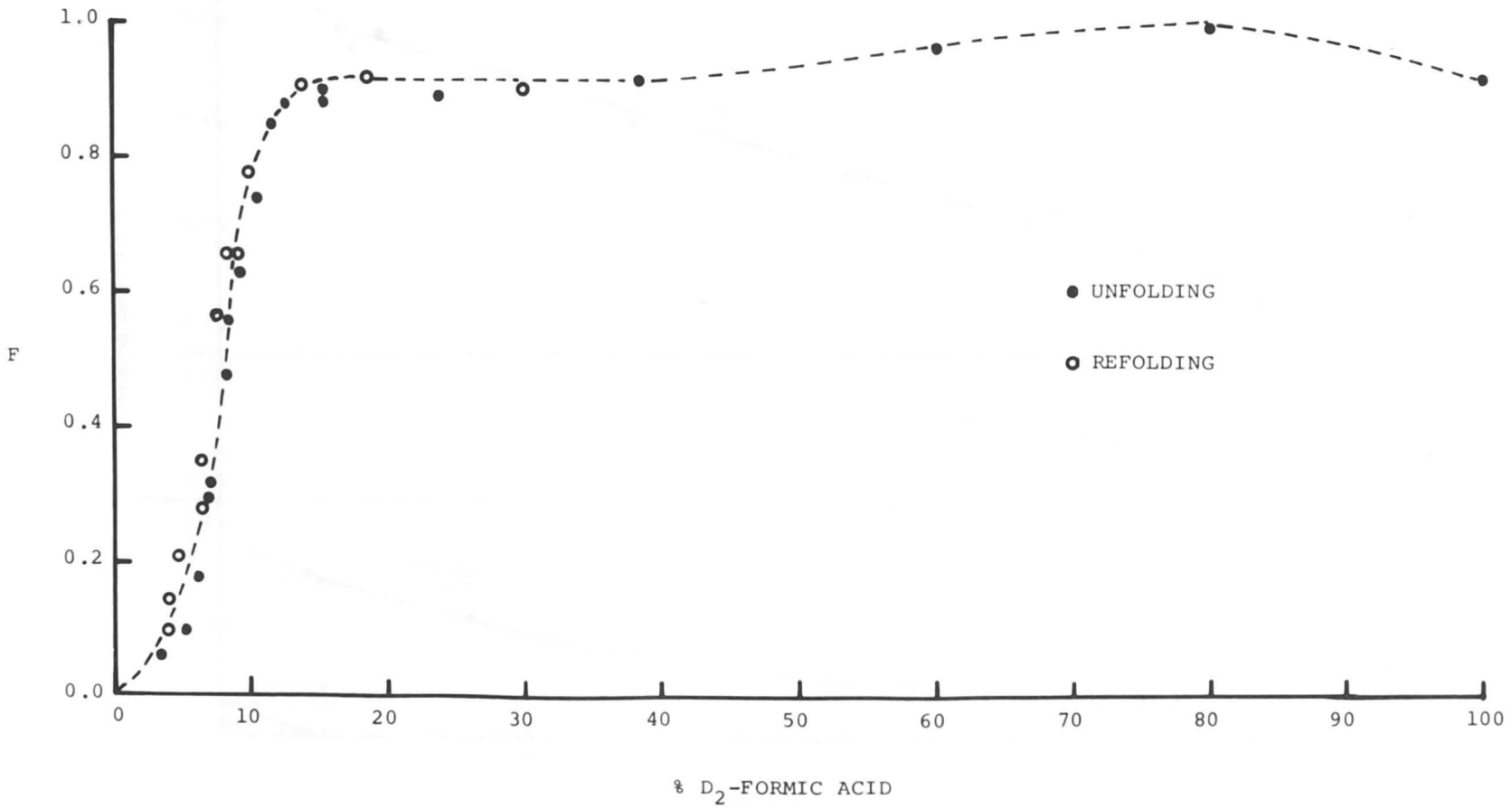
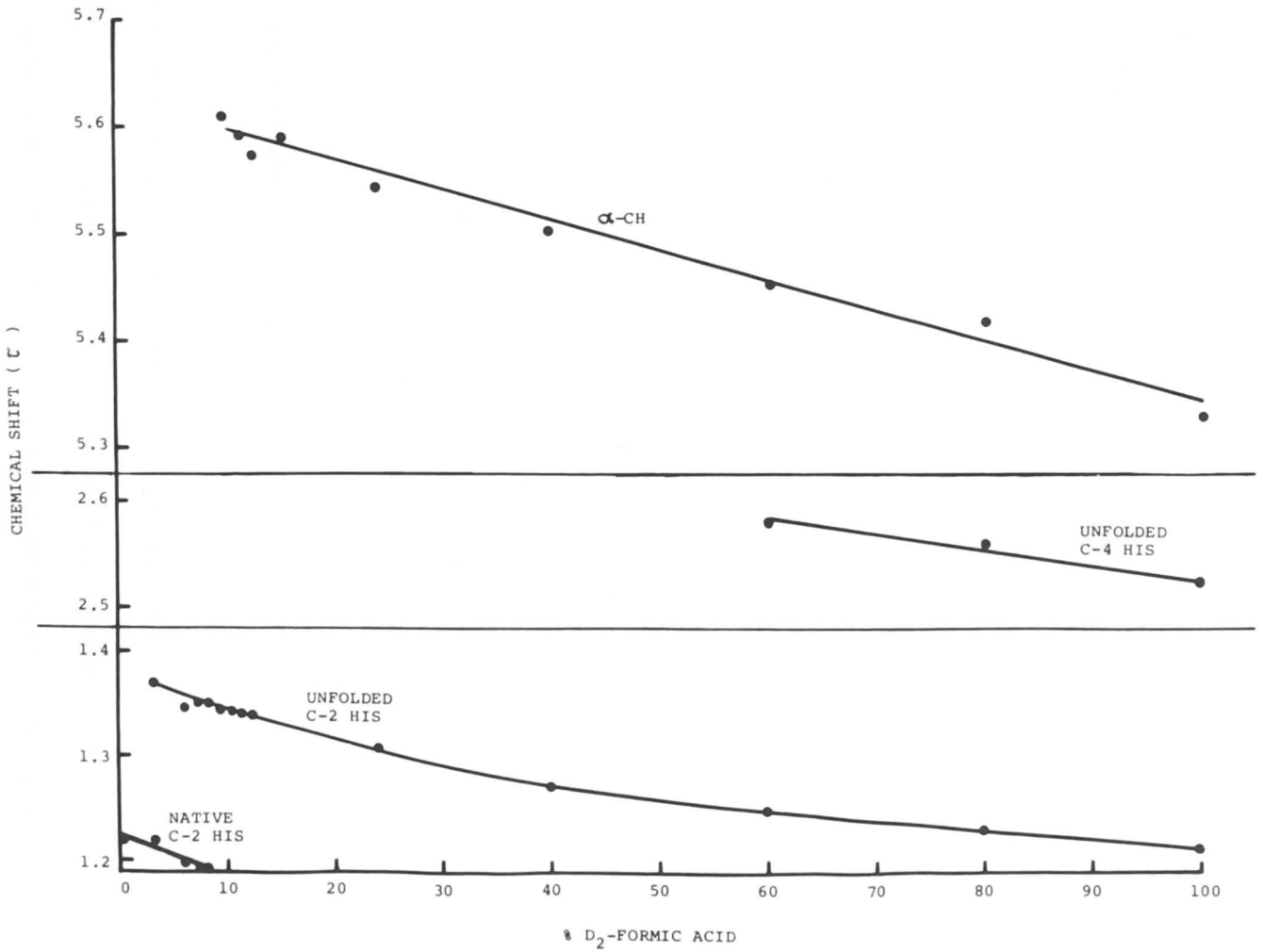


FIGURE 6.6

RIBONUCLEASE IN D_2-FORMIC ACID

HISTIDINE AND α-CH CHEMICAL SHIFTS



concentrations below 60% d_2-formic acid because it was then obscured by aromatic resonances.

6D Discussion

The presence of two methionine peaks in the transition region represents at least one intermediate stage in the unfolding, since the native spectrum has no methionine peak, while the completely unfolded state exhibits only one methionine peak (Figure 6.2). Of the two peaks in the transition region, the downfield one has a chemical shift equal to that of the completely unfolded conformation. As shown in Figure 6.4, the upfield peak reaches a maximum height at 10% d_2-formic acid, and then decreases in height as the concentration of d_2-formic acid is further increased. To give rise to the abnormal chemical shift of the upfield methionine peak, one or more methionine residues must be involved in non-covalent interactions with other amino acids in the intermediate conformation of ribonuclease. However, at this stage, it is not possible to specify which other residues are involved. If the methionine interactions involve only adjacent residues in the sequence, they would have less effect on the intermediate conformation of the enzyme molecule, than if residues remotely spaced in the sequence are involved.

Further evidence for intermediates is provided by Figure 6.5, where the transition curve consists of two steps. The major step, having a mid-point at 8% d_2-formic acid, is followed by a smaller transition at concentrations above 40% d_2-formic acid.

The NMR data do not rule out the possibility of a small proportion of aggregated molecules in equilibrium with unfolded molecules in anhydrous d_2-formic acid. Josefsson (1958) found that the inactivated ribonuclease, obtained by precipitation after treatment with anhydrous formic acid, consisted mainly of compact aggregates of MW 210,000, with much ordered structure. Such aggregates would give rise to very broad peaks in NMR spectra, and if present in small amounts, would be difficult to distinguish from a large proportion of unfolded molecules. The presence of a small proportion of aggregated molecules may account for the decrease in F at 100% d_2-formic acid below that at 80% (Figure 6.5). However, a large proportion of aggregates is not likely, since the corrected peak heights at maximum unfolding in d_2-formic acid were similar to those obtained with urea (exact agreement is not expected because of instrumental variations between the two series of NMR experiments). Thus, most of the aggregation reported

by Josefsson (1958) can be attributed to the precipitation step, rather than the formic acid treatment.

Josefsson (1962) reported similar aggregation for lysozyme treated in formic acid, and precipitated by ether. The NMR spectrum of this protein was also examined after successive additions of d_2-formic acid, as described for ribonuclease in section 6B. At acid concentrations of ca 15%, there was a time-dependent decrease in height of all peaks, showing that aggregation occurred at these intermediate concentrations of d_2-formic acid. However, in anhydrous formic acid, lysozyme was completely unfolded and disaggregated (Chapter 3).

Figure 6.5 also shows that, within experimental error, the major unfolding step is reversible, at least with respect to the variation in peak heights when the acid concentration is varied. Previous workers, Anfinsen et al. (1954), and Josefsson (1958), established that the loss in enzymatic activity was reversible.

Besides the calibration curve, obtained in the presence of ribonuclease, Figure 6.1 shows a plot of HDO chemical shift against formic acid concentration in the absence of protein. This plot indicates that the HDO peak is further downfield in the presence of protein

than in its absence. Such a downfield shift may be due to changes in hydrogen bonding, or to polarisation effects (e.g. von Hippel & Schleich, 1969). Furthermore, the downfield chemical shifts of the imidazole and α-CH peaks (Figure 6.6) may also be due to changes in hydrogen bonding with increasing concentrations of d_2-formic acid. The tendency of formic acid to form strong hydrogen bonds with peptide groups has been known for some time (e.g. Kendrew, 1954), and this undoubtedly contributes to the mechanism for unfolding by formic acid.

CHAPTER 7

THE DENATURATION OF RIBONUCLEASE BY POTASSIUM THIOCYANATE

7A Introduction

The denaturing action of potassium thiocyanate has been known since Hardy (1899) observed the formation of a gel after treatment of albumin in a solution of this salt for several days. More recently, von Hippel & Wong (1964) found that reduction of the thermal transition temperature of ribonuclease occurred at lower molar concentrations of potassium thiocyanate than of GuCl or urea. In this respect, potassium thiocyanate is a more effective denaturant. However, the action of potassium thiocyanate does not lead to a completely unfolded conformation (e.g. Tanford, 1968), and in this respect, potassium thiocyanate is less powerful than other denaturants.

7B Experimental

The progressive unfolding of Sigma ribonuclease by potassium thiocyanate at pH 8.0 was carried out as described in section 5B for the unfolding by urea.

7C Results and Discussion

In agreement with previous reports (e.g. Tanford, 1968), several aspects of the spectra of ribonuclease in potassium thiocyanate indicated that the protein was not completely unfolded in this denaturant. The minimum width of the methyl peak was 19 Hz, which is too large for a cross-linked random coil (Chapter 3). Furthermore, two methionine peaks were observed throughout the transition, as for the intermediate concentrations of formic acid.

In order to calculate values of F, h_U for each peak was taken as the maximum corrected height reached in the unfolding of ribonuclease by urea, the same h_U being used for both methionine peaks. Values of F for the C-2 histidine, phenylalanine, methionine and methyl peaks are listed in Table 7.1. Because of possible variations in instrumental conditions from one unfolding series to another, there is some uncertainty in the true value of each h_U, and consequently the F values in Table 7.1 should be regarded as relative rather than absolute.

The phenylalanine and methionine peaks underwent downfield chemical shifts of approximately 0.05τ per

TABLE 7.1

F VALUES FOR THE DENATURATION OF RIBONUCLEASE BY POTASSIUM THIOCYANATE AT pH 8.0

KSCN MOLARITY	F				
	C-2 HIS	PHE	DOWNFIELD MET	UPFIELD MET	METHYL
0.0	0.00	0.00	0.00	0.00	0.00
1.0	0.10	0.00	0.00	0.00	0.00
1.5	0.25	0.10	0.10	0.08	0.07
1.8	0.37	0.31	0.27	0.22	0.44
2.2	0.53	0.46	0.46	0.41	0.73
2.6	0.57	0.52	0.52	0.40	0.78
3.3	0.58	0.45	0.50	0.45	0.80
4.6	0.50	0.35	0.41	0.32	0.53
5.7	0.42	0.24	0.41	0.27	0.38

mole of added potassium thiocyanate. For the C-2 histidine peak, the downfield shift is illustrated in Figure 7.1.

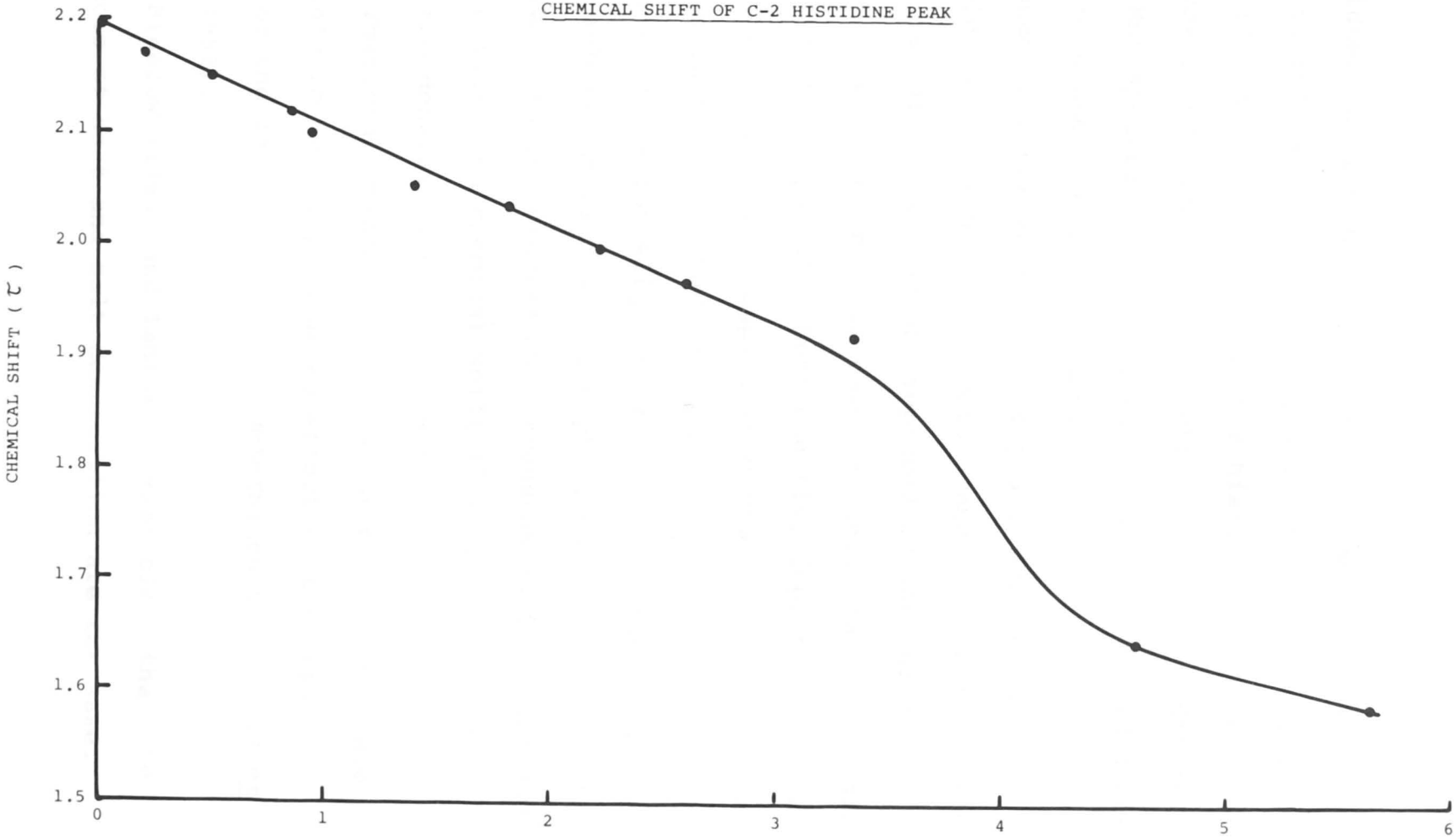
Table 7.1 shows that the denaturing action of potassium thiocyanate on ribonuclease at pH 8.0 consists of two stages. Firstly, there is an increase in peak heights, representing partial unfolding of the native molecules. This is followed by a decrease in peak heights, corresponding to either aggregation or a refolding. If refolding occurs, this would not be a direct return to native molecules, since the diminution in peak heights proceeds to different extents for different residues. Although the NMR data cannot be used to distinguish between refolding and aggregation, von Hippel and Wong (1965) found, by sedimentation equilibrium, that ribonuclease solutions were monomeric at high salt concentrations. Hence ribonuclease probably has a refolded non-native conformation at high potassium thiocyanate concentrations.

The refolding is accompanied by an additional downfield shift of the C-2 histidine peak in the region of 4M potassium thiocyanate, where the curve departs from its linear trend (Figure 7.1). The C-2 peak in spectra of native ribonuclease at pH 8.0 arises from

FIGURE 7.1

RIBONUCLEASE IN POTASSIUM THIOCYANATE

CHEMICAL SHIFT OF C-2 HISTIDINE PEAK



residues 12, 105 and 119; the C-2 peak of His 48 is not visible at pH >6 (Chapter 4). During the unfolding, the initially broad (12 Hz) C-2 histidine peak became narrower and increased in height. Since the width in a 100 MHz spectrum was 20 Hz, the large initial width was due to nonequivalence in chemical shift of the three resonances contributing to the peak. Unlike the unfolding at low pH's (Chapters 5 and 6), the composite C-2 histidine peak was not replaced by another at higher field when ribonuclease was unfolded by potassium thiocyanate at pH 8.0. Consequently, deprotonation of the three imidazole groups must change their environments from slightly perturbed to unperturbed configurations. Other evidence for slight conformational changes in ribonuclease at mild alkaline pH's, was obtained from optical rotation studies by Jirgensons (1958). However, as the titration chemical shift (1τ) is much larger than the denaturation one (0.15τ), the slight conformational changes occurring during titration are unlikely to have a pronounced effect on the apparent pK's of the imidazole groups, determined by Meadows et al. (1968).

Bigelow (1964) and Tanford (1968) claim that the products of heat and salt denaturation are the same.

However, spectra of heat-denatured ribonuclease (McDonald & Phillips, 1969) have a single rather than a double methionine peak. The non-covalent interactions giving rise to the upfield methionine peak are therefore absent in heat-denatured ribonuclease.

CHAPTER 8

THE DENATURATION OF LYSOZYME BY UREA

8A Introduction

Saunders & Wishnia (1958) were the first to publish an NMR spectrum of lysozyme. Subsequently, there have been numerous NMR studies of the thermal denaturation of lysozyme (Mandel, 1964; Ferguson & Phillips, 1967; McDonald & Phillips, 1967a & b; and Sternlicht & Wilson, 1967). However, the spectral changes have not been related to conformational changes in any precise manner.

Cohen & Jardetzky (1968) examined the effect of urea on lysozyme at pH 4.5. They reported that an NMR peak, attributed to tryptophan C-2 protons, moved downfield on the addition of urea. There was little other change in the spectrum, showing that no major conformational change had occurred. The viscosity studies of Glazer (1959) had previously demonstrated that the pH must be <3 or >8, in order to extensively unfold lysozyme in 8M urea. This was confirmed by NMR in Chapter 3.

Although there is no major conformational change, the enzymatic activity decreases at pH 7 on the addition of urea (Léonis, 1956). This may be related to the downfield shift of the tryptophan peak, since there are tryptophan residues in the cleft comprising the active site of lysozyme (Blake et al., 1967).

In the present work, an NMR study of the unfolding of hen egg-white lysozyme by urea was carried out at pH 2.8, since at this pH, the enzyme is completely unfolded (Chapter 3).

8B Experimental

The progressive unfolding of lysozyme by urea at pH 2.8 in H_2O (as well as in D_2O) was carried out as described in section 5B for ribonuclease. Following the unfolding series at 60 MHz, the experiment was repeated in H_2O on the 100 MHz spectrometer in order to follow the change in height of the lysine NH peak. After each addition of urea, and readjustment of pH, the spectrum was accumulated in the region -1 to 4τ for 1 hr (30 scans) at a sweep rate of 5 Hz per sec. A single scan of the spectrum at a sweep rate of 1 Hz per sec was then recorded in the region 6.5 to 11.0τ.

8C Results

The F values obtained from 60 MHz spectra for the unfolding of lysozyme in H_2O are shown in Figure 8.1. The C-2 histidine peak was not clearly visible in some of the H_2O spectra. This may have been due to overlapping peptide NH resonances, or to additional broadening in H_2O (Section 4C). The unfolding was therefore repeated in D_2O at pH 2.8. In spectra of native lysozyme, the C-2 histidine peak is displaced 0.35τ downfield from its position in spectra of the unfolded enzyme. In the transition region, both peaks are visible (Figure 8.2).

For the methyl and methionine peaks, the mid-point of the transition was at 4.8M urea in H_2O and at 5.0M in D_2O.

In contrast to ribonuclease, a slight precipitate was present at urea concentrations of 5.6M and above. After three hours in the spectrometer probe, the 8.5M solution in H_2O formed a gel.

F values obtained from the 100 MHz series are shown in Table 8.1. Because of the higher sensitivity and better resolution of this instrument, the heights of the lysine NH, alanine, threonine and a high-field methyl peak could also be followed throughout the

TABLE 8.1

F VALUES FOR THE UNFOLDING OF LYSOZYME BY UREA IN H_2O (pH 2.8)

UREA MOLARITY	F* PEPTIDE NH	LYS NH	ARG NH	MET	ALA	THR	METHYL	UPFIELD
0.0	0.00	0.00	0.00	0.0	0.0	0.0	0.0	0.0
1.8	0.00	0.00	0.10	0.0	0.0	0.0	0.0	0.0
2.7	0.00	0.09	0.21	0.0	0.0	0.0	0.0	0.1
3.4	0.06	0.07	0.27	0.0	0.0	0.0	0.0	0.1
4.1	0.21	0.21	0.55	0.3	0.4	0.4	0.2	0.3
4.7	0.78	0.72	0.99	0.8	0.7	0.7	0.7	0.7
5.3	0.88	0.88	1.00	0.9	0.9	0.9	0.9	0.9

* Obtained from 100 MHz spectra.

FIGURE 8.1

LYSOZYME IN UREA (pH 2.8)

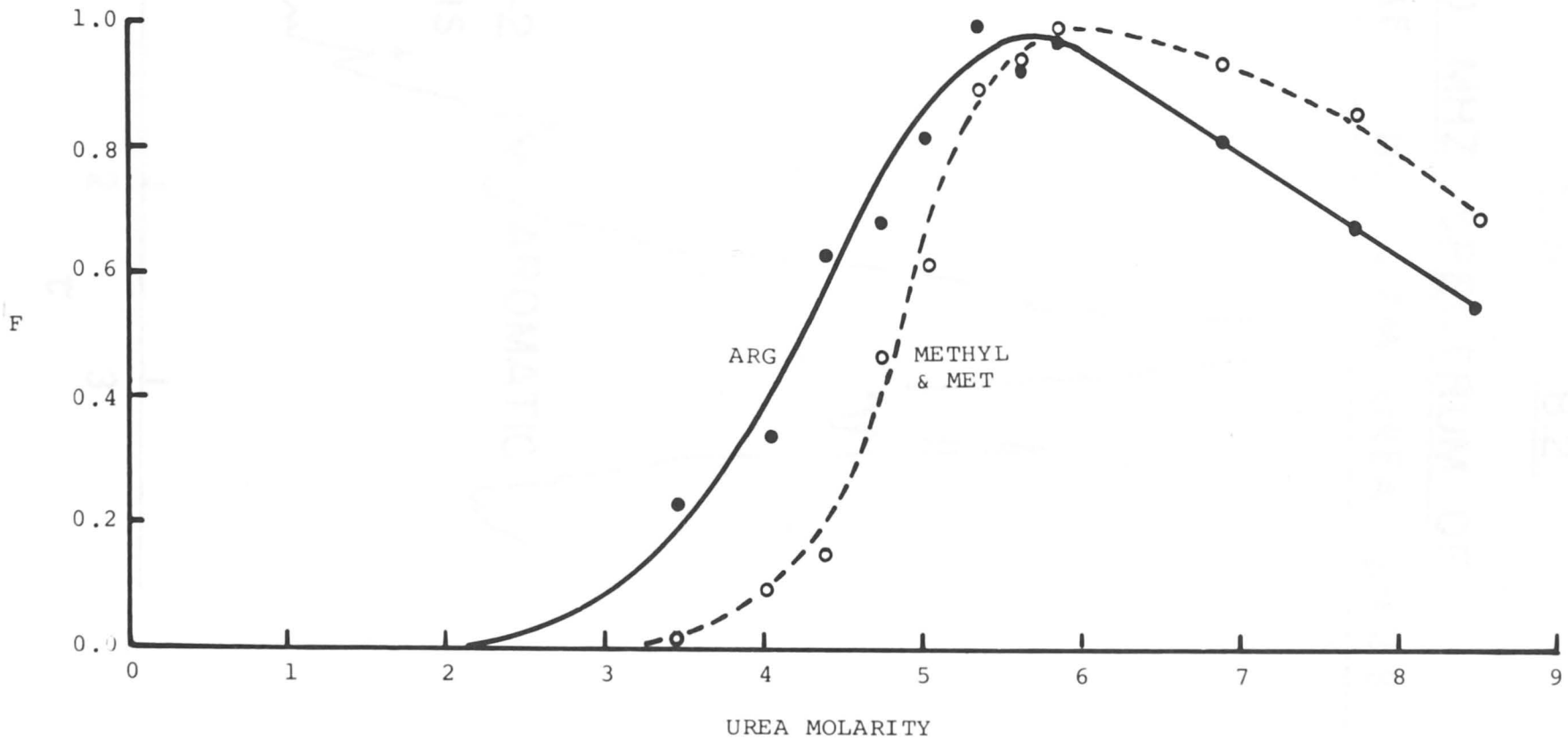
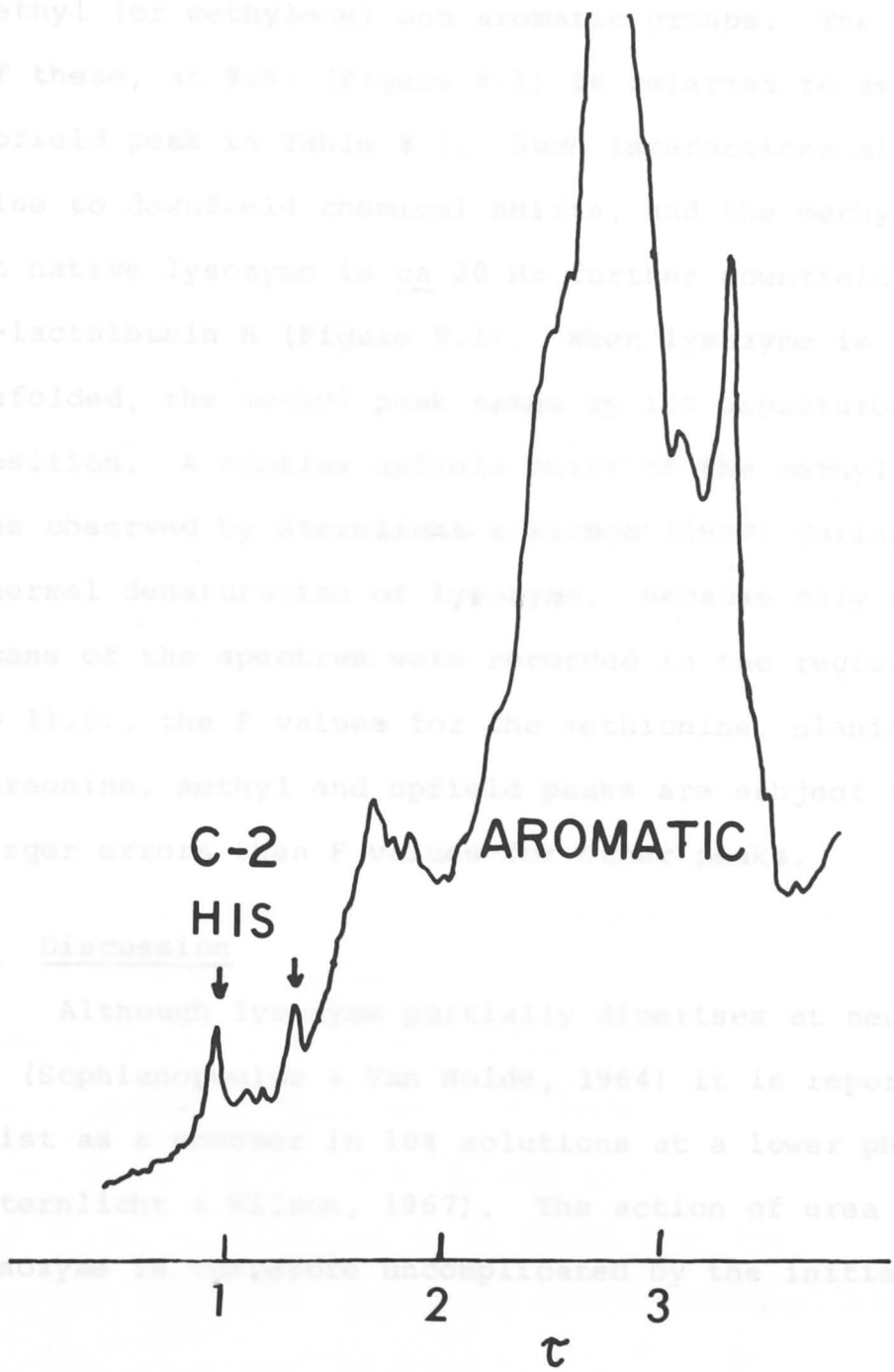


FIGURE 8.2

60 MHZ SPECTRUM OF LYSOZYME IN 4·7M UREA : pH 2·8



unfolding. The high-field resonances of lysozyme have been attributed by McDonald & Phillips (1967a & b) and Sternlicht & Wilson (1967) to interactions between methyl (or methylene) and aromatic groups. The largest of these, at 9.9τ (Figure 9.1) is referred to as the upfield peak in Table 8.1. Such interactions also give rise to downfield chemical shifts, and the methyl peak in native lysozyme is ca 20 Hz further downfield than in α-lactalbumin B (Figure 9.1). When lysozyme is unfolded, the methyl peak takes up its unperturbed position. A similar upfield shift of the methyl peak was observed by Sternlicht & Wilson (1967) during thermal denaturation of lysozyme. Because only single scans of the spectrum were recorded in the region 6.5 to 11.0τ, the F values for the methionine, alanine, threonine, methyl and upfield peaks are subject to larger errors than F values for other peaks.

8D Discussion

Although lysozyme partially dimerises at neutral pH (Sophianopoulos & Van Holde, 1964) it is reported to exist as a monomer in 10% solutions at a lower pH (Sternlicht & Wilson, 1967). The action of urea on lysozyme is therefore uncomplicated by the initial

dissociation which occurs with α-lactalbumin B (Chapter 9).

As shown in Figure 8.2, only two histidine peaks are observed during the unfolding, the downfield one corresponding to the native enzyme, and the second peak to the unfolded enzyme. The transition is therefore two-state with respect to the single histidine residue in lysozyme. However, this does not imply that the transition is two-state with respect to the whole molecule.

At any urea concentration within the transition region, Table 8.1 and Figure 8.1 show that the F value for the arginine NH peak is higher than F values for other peaks, which are all equal within experimental error.

According to the structure of crystalline lysozyme, as determined by X-ray diffraction (Blake et al., 1967) the acidic and basic side-chains are all on the surface of the molecule, while a number of nonpolar residues form a hydrophobic core. Furthermore, seven of the eleven arginine residues form three positively charged clusters on the surface (Browne et al., 1969). From the relative values of F, it seems that these arginine clusters unfold at lower urea concentrations than other

portions of the molecule. Partially unfolded lysozyme molecules are therefore present as intermediates throughout the unfolding transition which is clearly not a two-state process.

Since changes in optical rotation followed first order kinetics, over a range of temperature and pH, Aune & Tanford (1969) concluded that the unfolding of lysozyme by GuCl is a single-step process. However, just as the height of the NH peak is not measurably altered by the unfolding of the arginine clusters, so the optical rotation may not be significantly affected. Other methods may not detect intermediates of the type revealed by the non-equivalent F values for the unfolding of lysozyme by urea. Unfortunately, in the unfolding by GuCl, such intermediates (if present) could not be detected by NMR, since the guanidyl NH peak obscures the arginine NH peak in 60 MHz spectra.

The decrease in peak heights at urea concentrations greater than 6M, as well as the formation of a gel in the 8.5M solution after a few hours, shows that a time-dependent aggregation of the unfolded molecules follows the unfolding steps.

When lysozyme was unfolded in an 8M urea solution, and the urea removed by dialysis before gelation, the

NMR spectrum returned to its native form. This shows that the unfolding steps are essentially reversible.

α-Lactalbumin B is believed to have a similar structure to that of hen egg-white lysozyme (Browne et al., 1969). The unfolding of these two proteins by urea is compared in the next chapter.

CHAPTER 9

THE ACTION OF UREA ON α-LACTALBUMIN B*

9A Introduction

Lysozyme and α-lactalbumin B have similar amino-acid sequences (Brew et al., 1967). In view of this similarity, Browne et al. (1969) recently proposed a possible three-dimensional structure for α-lactalbumin B, in which the homologous side-chain interactions were assumed to be the same as those found for lysozyme by X-ray diffraction (Blake et al., 1967).

Support for the proposed structure may be found in the solvent perturbation studies of Kronman & Holmes (1965) and the titration studies of Robbins et al. (1967). The two proteins have different absorptions in the aromatic regions of their ultraviolet spectra, but these have been attributed to differences in amino-acid composition (Browne et al., 1969).

The optical rotatory dispersion parameter, b_o, as determined by Herskovits & Mescanti (1965), differs considerably between lysozyme (-150) and α-lactalbumin B

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* α-Lactalbumin A, a genetic variant, has the same amino acid sequence except that Gln replaces Arg 10 (Hopper, 1970).

(-235). This difference was attributed by Kronman (1968) to differences in optical activity of the side-chains, rather than to differences between the backbone conformations of the two proteins. The same conclusion was reached from circular dichroism studies (Kronman, 1968).

On the other hand, Atassi et al. (1970) found that lysozyme and α-lactalbumin B exhibit differences in immunochemical reactions, as well as differences in accessibility of disulphide bonds to reduction. These results were interpreted in terms of appreciable variations in conformation between the two proteins.

In order to obtain further information on the structure of α-lactalbumin B, an NMR study of its denaturation by urea was undertaken. Hopper (1970) carried out optical rotation studies on the same sample of protein in urea solutions, and this provides an opportunity to compare the NMR results with those obtained by another technique.

9B Experimental

I am grateful to Mr. K.E. Hopper for supplying a chromatographically purified sample of bovine α-lactalbumin B. The preparation of the protein, which

was homogeneous on starch-gel electrophoresis, is described by Hopper (1970) and Armstrong et al. (1970).

The progressive urea-denaturation of α-lactalbumin B, monitored by the 60 MHz spectrometer, was carried out at pH 2.5 in D_2O as described in section 5B for the unfolding of ribonuclease, except that for some spectra, fewer scans were accumulated. In the absence of urea, the protein solution formed a gel after five hours in the NMR tube, but on the addition of a small amount of D_2O and urea, with stirring, the gel liquefied. There was no evidence for aggregation at high urea concentrations.

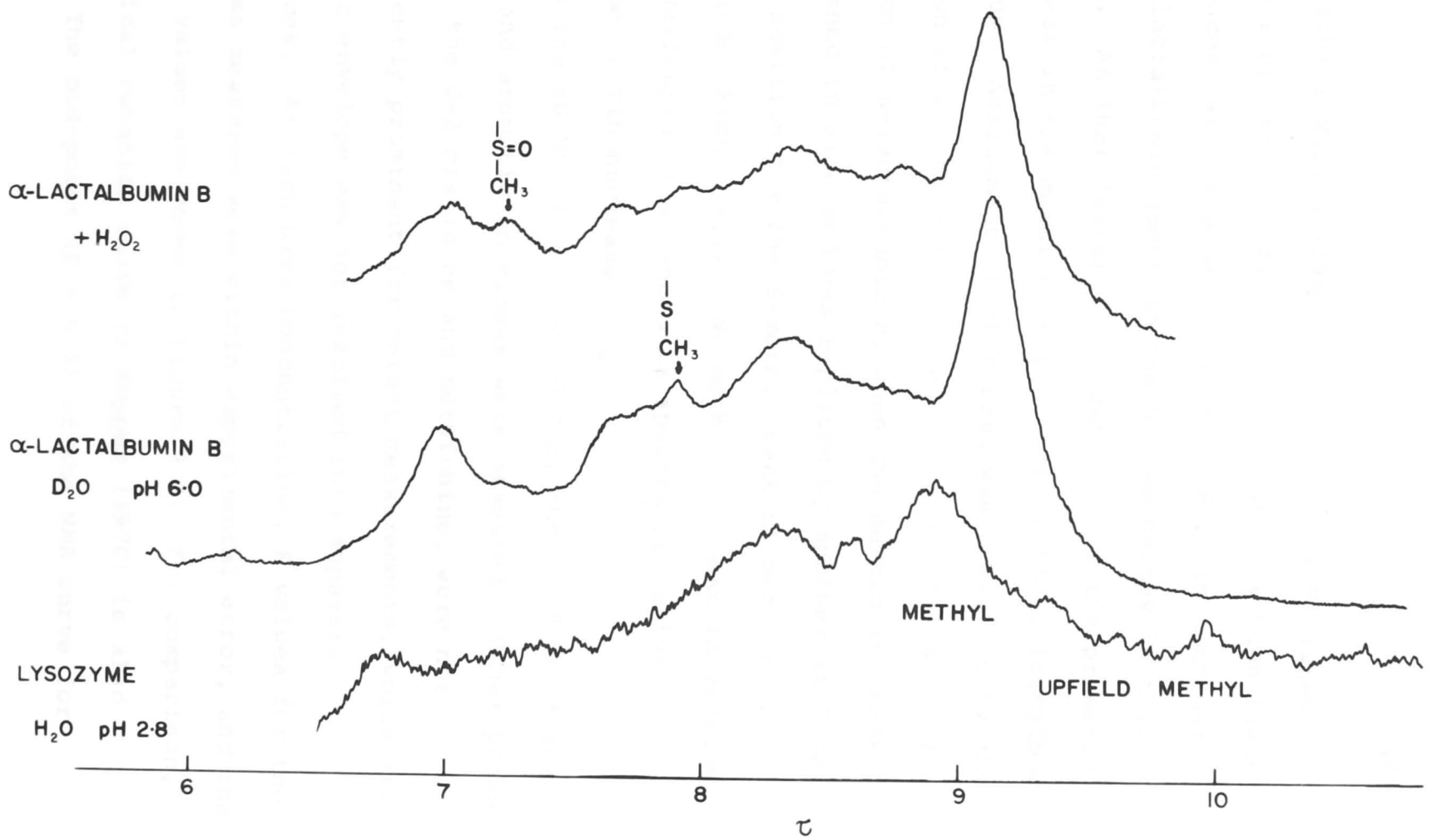
For the series in D_2O at pH 6.0, the 100 MHz spectrometer was used. After each addition of urea, and readjustment of pH, 30 scans were accumulated in the region -1τ to 4τ, and 15 scans from 6.5τ to 11.0τ, both at a sweep rate of 5 Hz per sec.

9C Results

100 MHz spectra of native lysozyme and α-lactalbumin B, on the upfield side of the HDO peak, are shown in Figure 9.1. There is a higher signal-to-noise ratio in the α-lactalbumin spectrum, as it represents the average of 15 scans, whereas the lysozyme spectrum is only a single scan. In agreement with previous reports by

FIGURE 9.1

100 MHZ SPECTRA OF LYSOZYME & α-LACTALBUMIN B

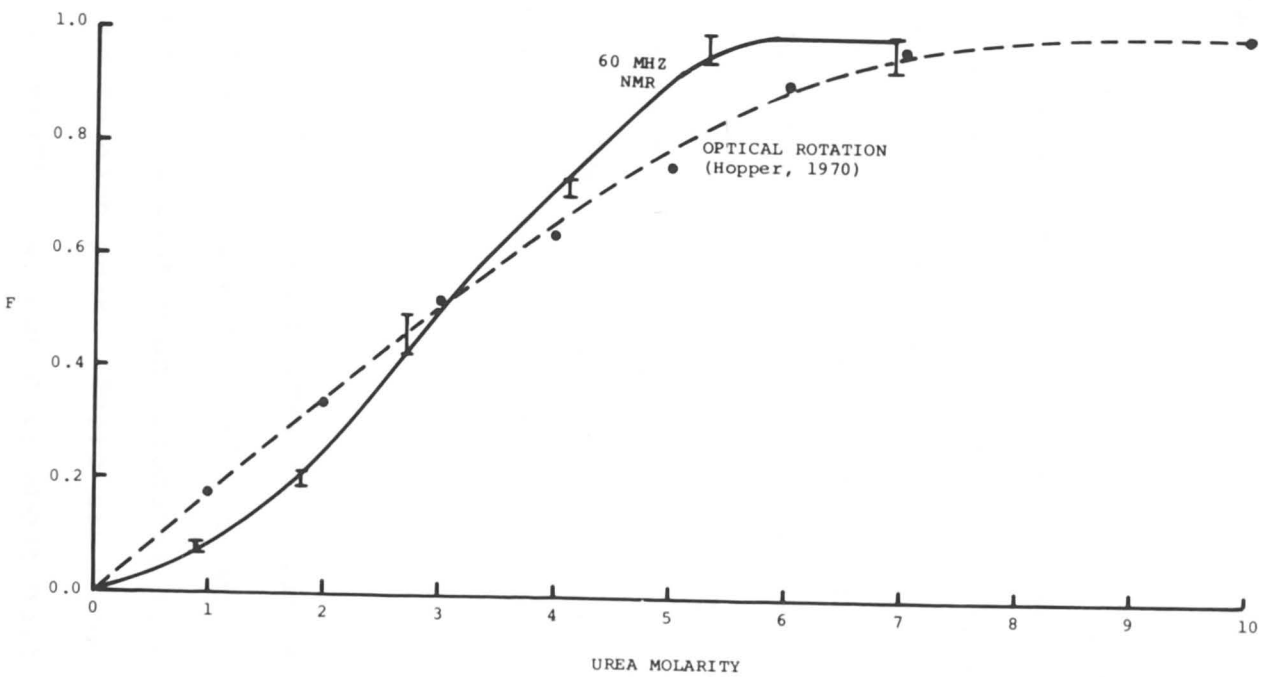


Sternlicht & Wilson (1967) and McDonald & Phillips (1967a & b), the lysozyme spectrum exhibits high-field resonances, as discussed in section 8C. In contrast, the α-lactalbumin spectrum has no resonances in this region. Another feature of Figure 9.1 is the presence of a peak in the methionine position in the α-lactalbumin spectrum. Assignment of this peak was confirmed by the addition of 0.1 ml 30% hydrogen peroxide to 0.45 ml 10% solution of α-lactalbumin B, when the methionine peak diminished in size and was replaced by another at 7.22τ in the position for the S-methyl peak of methionine sulphoxide (Figure 9.1). No methionine peak is resolved from overlapping resonances in spectra of native lysozyme or ribonuclease (Figure 5.1).

In the 60 MHz series at pH 2.5, the heights of the methyl and aromatic envelopes were measured. Other peaks, such as the C-2 histidine and methionine, were not sufficiently prominent for height measurements, while the aromatic envelope was not resolved into separate resonances. At each urea concentration, F values for the two peaks measured were within experimental error, and the average values are shown in Figure 9.2. For comparison, the optical rotation curve of Hopper (1970) is also shown. The mid-point (F = 0.5) of the NMR curve for

FIGURE 9.2

α-LACTALBUMIN B IN UREA (pH 2.5)



Vertical lines represent total range in F over both peaks measured

α-lactalbumin B (pH 2.5) is at 3.0M urea, while that for the methyl and methionine peaks of lysozyme (pH 2.8) is at 4.9M urea (Figure 8.1).

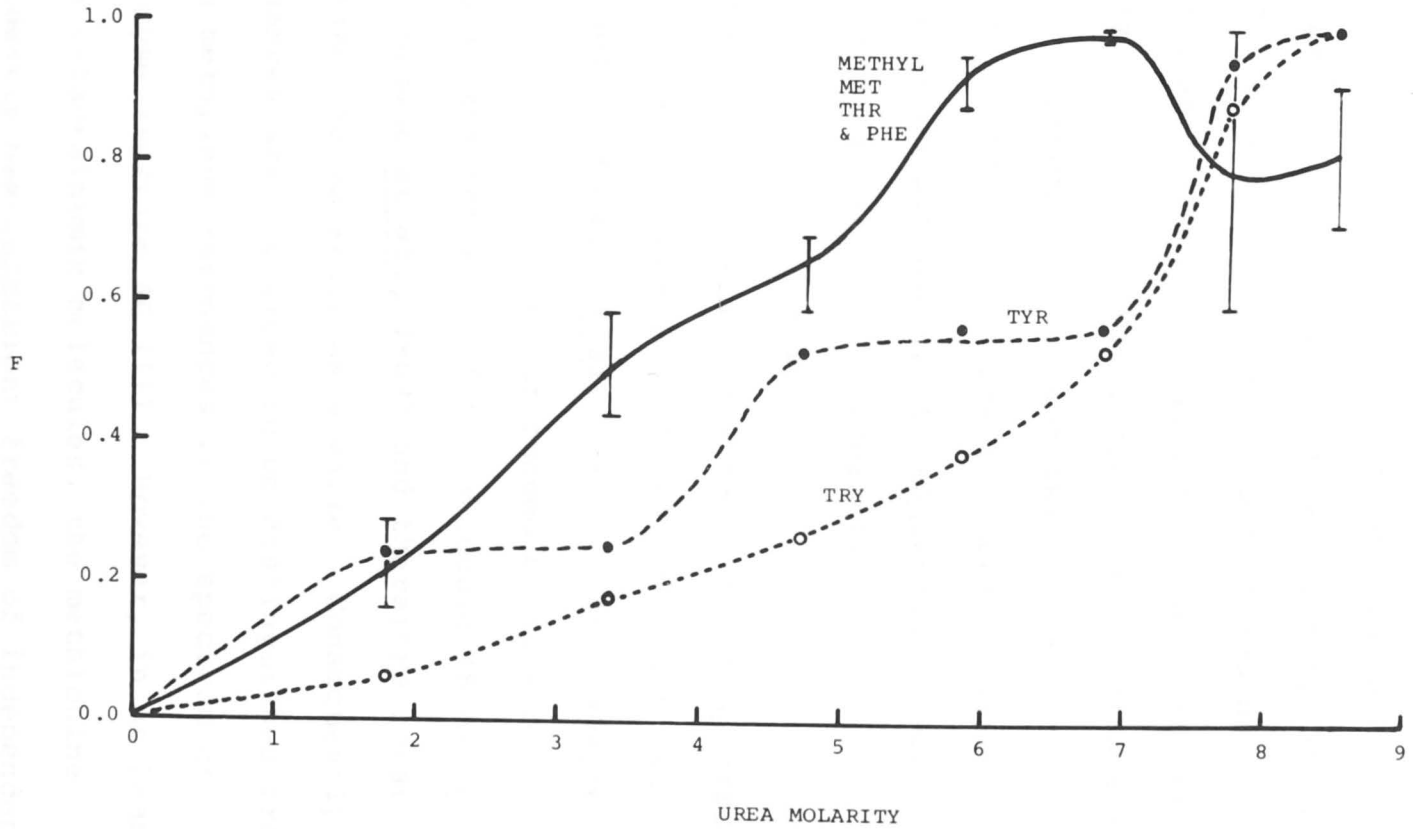
Figure 9.3 summarises results obtained at pH 6.0 on the 100 MHz spectrometer. At each concentration below 7M, the phenylalanine, methionine, threonine and methyl peaks all had F values which were equal within experimental error. The average values for these peaks are shown as a single curve in Figure 9.3, with each vertical line representing the total range in F at the corresponding urea concentration. F values for a peak arising from the C-4 and C-7 indole protons of tryptophan, as well as another peak due to the C-2 and C-6 aromatic protons of tyrosine, are also shown in Figure 9.3. These two peaks were assigned as in Cohen & Jardetzky (1968).

9D Discussion

An analysis of the upfield resonances in lysozyme has been given by Sternlicht & Wilson (1967) in terms of the particular interacting aliphatic and aromatic side-chains which contribute to each peak. There are more than twenty of these interactions, but only about half of them are retained in the proposed model of

FIGURE 9.3

α-LACTALBUMIN B IN UREA (pH 6.0)



Vertical lines represent total range in F over all peaks measured.

α-lactalbumin B. The 100 MHz spectrum of native α-lactalbumin B in Figure 9.1 lacks upfield peaks. However, a recent 220 MHz spectrum reported by Cowburn et al. (1970) shows several small upfield resonances, with chemical shifts roughly equal to those obtained by calculations based on the predicted model of Browne et al. (1969). Furthermore, in contrast to lysozyme, α-lactalbumin B showed no change in chemical shift of the methyl peak on unfolding. Consequently, there are many fewer aromatic-aliphatic interactions in α-lactalbumin B than in lysozyme.

The presence of a methionine peak in the spectrum of α-lactalbumin B (Figure 9.1) is in agreement with the proposed model of Browne et al. (1969). In lysozyme, both Met 12 and Met 105 are surrounded by numerous other side-chains in the interior of the molecule (Blake et al., 1967; Browne et al., 1969) and therefore rotate no faster than the molecule as a whole. Consequently, their resonances are too broad to be distinguished from overlapping methylene resonances in the spectrum of native lysozyme (section 4C (i)). However, in at least some of the α-lactalbumin molecules, the methionine residue evidently has sufficient freedom of independent movement for its S-methyl peak to be observed, with a

width of 9 Hz. This agrees with the proposed structure of Browne et al. (1969), in which the side-chain of Met 90 projects into the surrounding liquid.

The formation of a gel and the extremely small peak heights, in α-lactalbumin B at pH 2.5, suggest the occurrence of a time-dependent aggregation. This confirms an earlier report of aggregation in α-lactalbumin B at acid pH's (Kronman & Andreotti, 1964). Furthermore, Kronman et al. (1964) and Robbins et al. (1967) found that a slight conformational change preceded aggregation when the protein was titrated below pH 4. Consequently, the transition curves in Figure 9.2 reflect all the changes involved in passing from aggregated α-lactalbumin B to the unfolded conformation. The unfolding transition for lysozyme represents only the change from native monomer to completely unfolded protein. Undoubtedly, the accompanying disaggregation contributes to the much broader transition observed for α-lactalbumin B (Figures 8.1 and 9.2).

The lysozyme and α-lactalbumin transitions also differ in that the latter protein unfolds at lower urea concentrations. Furthermore, Atassi et al. (1970) reported that lysozyme was more resistant to denaturation by GuCl. It seems reasonable to suggest that the

greater abundance of aromatic-aliphatic interactions in lysozyme, confers additional stability on this enzyme.

As shown in Figure 9.2, both the NMR and optical rotation curves indicate that the transition occurs over the same range of urea concentrations, except that the optical rotation curve extends to higher denaturant concentrations. Both curves indicate a mid-point for the transition at 3.0M urea.

Although aggregation is minimal at pH 6.0, not all molecules are monomeric in 8% solutions of α-lactalbumin B at this pH (Kronman & Andreotti, 1964). As at pH 2.5, the transition in Figure 9.3 at pH 6.0 therefore involves dissociation in addition to unfolding. The different paths followed by the tryptophan, tyrosine and average curves in Figure 9.3 reflect the multi-step nature of the transition. Not all peaks reach their maximum height at the same urea concentration, showing that at no stage are the molecules completely unfolded. Further evidence for incomplete unfolding arises from the lack of a single sharp C-2 histidine peak at high urea concentrations. Kronman & Holmes (1965) previously showed by solvent perturbation methods that the tryptophan residues were not completely exposed in 8M urea. Nevertheless, α-lactalbumin B is extensively

unfolded both at pH 2.5 and pH 6.0 (as indicated by the widths of the methyl and other peaks). This may be contrasted with the pH dependence for lysozyme, which is only slightly unfolded at pH 6.0, but completely unfolded at pH 2.5 (Figures 3.4 and 3.5).

During the transition at pH 6.0, the methionine peak quadrupled in height, but its width underwent a correspondingly smaller change (from 9 to 6 Hz). The apparent increase in area indicates that not all Met 90 residues have sufficient freedom of movement to contribute to the methionine peak in the native spectrum. This may arise from the existence of more than one possible conformation for Met 90 in native α-lactalbumin B, or it may be due to the association mentioned above.

Because of the absence of numerous upfield peaks, the reduced stability towards denaturation by urea, and the different pH dependence of the unfolding, many of the side-chain interactions in native α-lactalbumin B must be different from those in lysozyme. However, numerous interactions, different from those in lysozyme, exist in the proposed model for α-lactalbumin B (Browne et al., 1969). Thus, none of the results reported in this Chapter are necessarily inconsistent with the proposed model.

CHAPTER 10

CONCLUSIONS AND SUGGESTIONS FOR FURTHER APPLICATIONS OF NMR METHODS

10A Denaturation

Although little has been contributed to the question of the mechanism for denaturation of proteins, this thesis has shown how NMR may help in the characterisation of the denatured state, and also in determining whether or not stable intermediates are present in an unfolding transition.

The method outlined in Chapter 3 for detecting residual non-covalent interactions in proteins (i.e. by simply examining the NMR spectrum for abnormal chemical shifts, widths or areas) should be applicable to any protein-denaturant system. Small regions of ordered structure, to which hydrodynamic methods are insensitive (Miller & Goebel, 1968), can be detected by the NMR method if the groups involved in non-covalent interactions are those which give rise to prominent peaks in spectra of completely unfolded proteins. An example is provided by Figure 5.7, where one of the

methionine residues in unfolded ribonuclease gives rise to a peak slightly further downfield in H_2O than in D_2O.

Furthermore, intermediate stages in an unfolding transition can be recognised by (i) the appearance of additional peaks not corresponding to either native or unfolded molecules or (ii) non-equivalent F values among all peaks. As noted in Chapters 5-9, intermediates are present in most of the transitions studied in this thesis.

10B NMR Difference Spectra

Selective deuteration is one method for reducing the complexity of macromolecular NMR spectra (e.g. Crespi & Katz, 1969), but the production of deuterated proteins is not always feasible. An alternative procedure is that of NMR difference spectroscopy, where peaks whose presence or chemical shift is dependent on one parameter, may be separated from other peaks which are not dependent in the same manner. Bak et al. (1967) used this technique to examine differences between the spectra of bovine and porcine insulin in TFA. In Figure 5.7, the NH resonances of lysine and arginine side-chains were separated from aromatic resonances by subtracting the D_2O spectrum from the H_2O one. Similarly in Chapter 4,

by means of NMR difference spectroscopy, the C-4 imidazole peaks of ribonuclease were separated from the aromatic resonances of phenylalanine and tyrosine by varying the pH. Examination of the C-4 peaks in the presence of inhibitors may yield further information on the enzymic mechanism of ribonuclease. This would supplement data obtained from the C-2 peaks by Meadows et al. (1969).

10C Inter-nuclear Distances

NMR has been extensively used for measuring inter-nuclear distances in crystals (e.g. Richards, 1956), but not in solution. Nevertheless, Kayne & Reuben (1970) have recently determined the distance between the mono- and di-valent binding sites of pyruvate kinase by thallium-205 nuclear magnetic resonance. While novel, their method is restricted to metal-binding sites on proteins. A technique of wider applicability would be one which measured inter-proton distances in solution.

Equation (1) shows that the dipolar interaction is inversely proportional to the sixth power of the inter-proton distance, and is consequently dominated by nearest-neighbour protons. In proteins, most nearest-neighbour protons are located on the same chemical group

(e.g. the protons of a methyl group) and the proximity of another chemical group causes little additional broadening.

However, the nearest imidazole proton to the C-2 proton of histidine in D_2O is the C-4 proton, 4.25 °A away. This is not sufficiently close to cause any appreciable broadening for an imidazole group in a protein of the size of chymotrypsin. Consequently, the proximity of a proton on a nearby group in the native protein may cause pronounced broadening. Using equation (1), the inter-proton distance could be estimated from the imidazole peak width. In addition to being important for structural studies of proteins, a knowledge of distances between imidazole protons and nearest-neighbour protons would be valuable in determining the mode of binding of an inhibitor to a protein such as ribonuclease with histidine residues at its active site. However, before applying this method, other mechanisms for broadening, e.g. exchange, would need to be ruled out.

Since the C-4 imidazole proton of histidine and the C-2 indole proton of tryptophan are similarly remote from protons on the same amino acid, inter-nuclear

distances involving these protons might also be measured by NMR.

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APPENDIX

DEPENDENCE OF PEAK WIDTH ON DP FOR HELICAL POLYPEPTIDES

Bradbury & Stubbs (1968) and Chapman (1968) have measured the widths of peaks arising from side-chain protons in helical polypeptides. In agreement with earlier results for polyethylene glycol (Chujo et al., 1963), widths were found to increase with DP (degree of polymerisation). Stubbs (1967) developed a theoretical relationship between peak width and DP, but this relationshp predicted a much heavier dependence of width on DP than was observed experimentally.

In the following treatment, expressions derived by Woessner (1962) for the anisotropic motion of ellipsoids are applied to the side-chain groups of helical polypeptides. For assistance in reading this paper, I am grateful to Mr R.K. Milne.

For two protons separated by a constant distance b(°A), the spin-spin relaxation time is given by:-

$$\frac{1}{T_2} = 1.66 \times 10^{11} b^{-6} \left[J_o(0) + 10 J_1(\omega_o) + J_2(2\omega_o) \right] \qquad \text{(i)}$$

where the $J_h(\omega)$ are the Fourier intensities at frequency

ω of certain functions of the orientation of the vector joining the two nuclei.

For an assembly of N protons, where N > 2,

$$\frac{1}{T_2} = N^{-1} \sum_{i>j} \left(\frac{1}{T_2}\right)_{ij}$$

In order to evaluate the $J_h(\omega)$ for two protons fixed in an ellipsoid, Woessner (1962) chose a rectangular coordinate system with axes X, Y and Z coinciding with three axes of the ellipsoid. The internuclear vector has direction cosines l, m, and n with the axes X, Y and Z respectively. R_1, R_2 and R_3 are rotational diffusion coefficients of the ellipsoid about X, Y and Z respectively.

The result is (Woessner, 1962):-

$$J_h(\omega) = K_h\left[\frac{C_+\tau_+}{1+\omega^2\tau_+^2} + \frac{C_-\tau_-}{1+\omega^2\tau_-^2} + \frac{C_1\tau_1}{1+\omega^2\tau_1^2} + \frac{C_2\tau_2}{1+\omega^2\tau_2^2} + \frac{C_3\tau_3}{1+\omega^2\tau_3^2}\right]$$

where $K_o = \frac{4}{5}$; $K_1 = \frac{2}{15}$; $K_2 = \frac{8}{15}$

$$\frac{1}{\tau_1} = 4R_1+R_2+R_3 \; ; \quad \frac{1}{\tau_2} = 4R_2+R_1+R_3 \; ; \quad \frac{1}{\tau_3} = 4R_3+R_1+R_2$$

$$\frac{1}{\tau_+} = 6\left[R+\sqrt{R^2-L^2}\right] \; ; \quad \frac{1}{\tau_-} = 6\left[R-\sqrt{R^2-L^2}\right]$$

$$C_1 = 6m^2n^2 \; ; \quad C_2 = 6l^2n^2 \; ; \quad C_3 = 6l^2m^2$$

$$C_+ = d-e \; ; \quad C_- = d+e$$

and where

$$R = \frac{1}{3}(R_1+R_2+R_3) \; ; \quad L^2 = \frac{1}{3}(R_1R_2+R_2R_3+R_3R_1)$$

$$d = \frac{3}{2}(l^4+m^4+n^4) - \frac{1}{2}$$

$$e = \frac{1}{6}\left\{\delta_1(3l^4+6m^2n^2-1) + \delta_2(3m^4+6l^2n^2-1) + \delta_3(3n^4+6l^2m^2-1)\right\}$$

$$\delta_i = \frac{R_i-R}{\sqrt{R^2-L^2}}$$

For the purpose of this calculation, the relevant side-chain group of a helical polypeptide is considered as an ellipsoid. The X axis is taken as the line drawn perpendicular to the helical backbone and through the centre of the side-chain group. R_1 may be large if there is a low energy barrier for rotation of the side-chain group around a bond between it and the helix.

For the case where $R_1 >> R_2$ and $R_1 >> R_3$,

$$\frac{1}{\tau_+} \simeq 4R_1 \; ; \quad \frac{1}{\tau_-} \simeq 3(R_2+R_3)$$

$$J_h(\omega) \simeq K_h\left[\frac{C_-\tau_-}{1+\omega^2\tau_-^2}\right]$$

$$\delta_1 \simeq 2 \; ; \quad \delta_2 \simeq -1 \; ; \quad \delta_3 \simeq -1$$

and $C_- \simeq 0.5(3l^2-1)^2$

For either the benzyl protons of poly-γ-benzyl-L-glutamate, or the S-methyl protons of poly-L-methionine,

$l = 0$, b = 1.8 °A, and substitution in equation (i) yields the following expression for W_d (the dipolar contribution to the width).

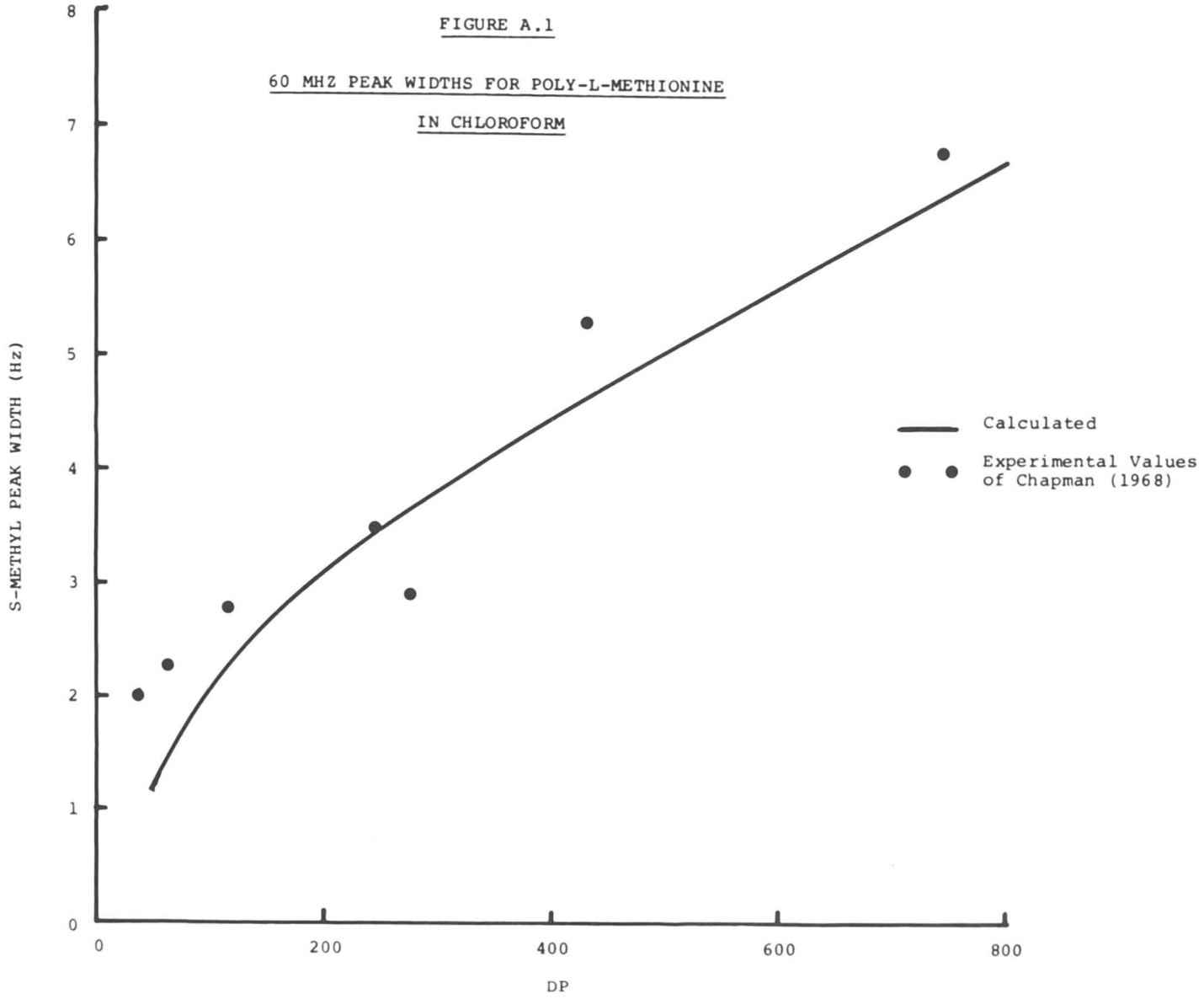
$$W_d = \frac{1.66 \times 10^{11} \tau_-}{2\pi\ 34} \left[\frac{4}{5} + \frac{4}{3(1+\omega_o^2 \tau_-^2)} + \frac{8}{15(1+4\omega_o^2 \tau_-^2)} \right] \qquad \text{(ii)}$$

$(R_2+R_3)^{-1}$ is the relaxation time for rotation of the X axis about the other two axes (e.g. Tanford, 1961). However, rotation of the helix controls rotation of the X axis for which the relaxation time is also $(R_1'+R_2')^{-1}$ where R_1' is the rotational diffusion coefficient for rotation of the helix about its major axis and R_2' corresponds to rotation about a minor axis. In shape, the helix resembles a long prolate ellipsoid for which R_1' and R_2' are quoted by Woessner (1962).

For poly-L-methionine in chloroform the parameters η = 0.005, minor semi-axis = 5 °A, and major semi-axis = 0.5(DP) were substituted in the expressions for R_1' and R_2' given by Woessner (1962). The resultant values of τ_- gave values for W_d by means of equation (ii). In Figure A.1 the calculated widths are compared with the experimental values determined by Chapman (1968).

FIGURE A.1

60 MHZ PEAK WIDTHS FOR POLY-L-METHIONINE IN CHLOROFORM



For poly-γ-benzyl-L-glutamate in dimethylformamide, $\eta = 0.008$, and the minor semi-axis is 7 °A. Calculated values for the width of the benzyl peak, together with the experimental results of Stubbs (1967), are shown in Figure A.2.

In view of the inherent approximations, the extent of agreement between the calculated and experimental widths is reasonable. The departure from linearity in both curves may be attributed to $\omega_o\tau_-$ being ca unity for much of the relevant range in DP.

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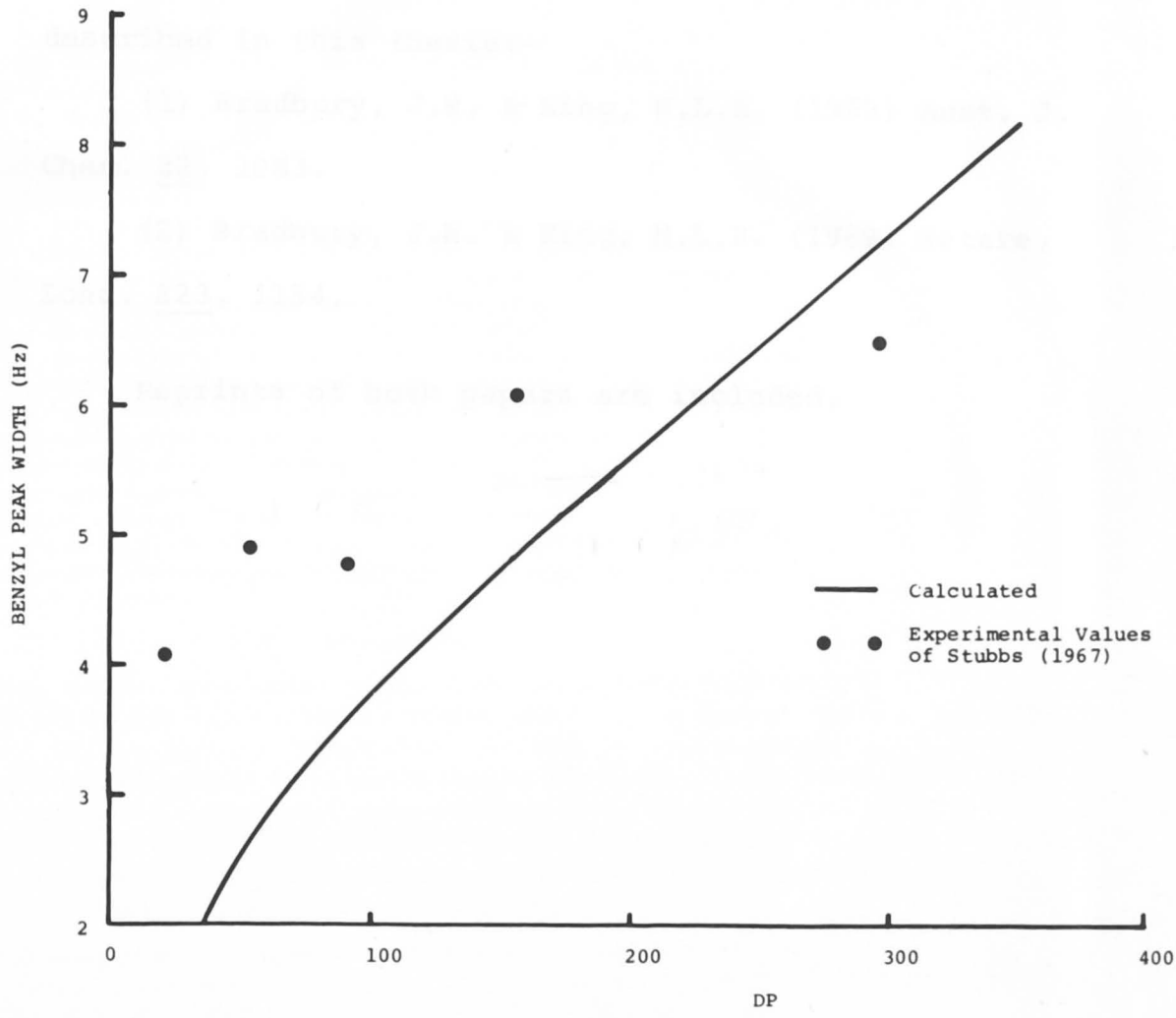
FIGURE A.2

60 MHZ PEAK WIDTHS FOR POLY-γ-BENZYL-L-GLUTAMATE IN DIMETHYLFORMAMIDE



PUBLICATIONS

The following publications were derived from work described in this thesis:-

(1) Bradbury, J.H. & King, N.L.R. (1969) Aust. J. Chem. 22, 1083.

(2) Bradbury, J.H. & King, N.L.R. (1969) Nature, Lond. 223, 1154.

Reprints of both papers are included.

Reprinted from the
AUSTRALIAN JOURNAL OF CHEMISTRY

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF DENATURED PROTEINS

By J. H. Bradbury* and N. L. R. King*

[Manuscript received November 19, 1968]

Summary

The proton magnetic resonance spectroscopy of 11 proteins (molecular weight range 5700–650000) has been investigated in five denaturing solvents, viz., trifluoroacetic acid-*d*, formic acid, dichloroacetic acid, 6M guanidine hydrochloride in D_2O, and 8M urea in D_2O. The chemical shifts, line-widths, and intensities of the resonances have been measured of the histidine C 2 protons, the methionine SCH_3 protons and methyl protons of leucine, isoleucine, and valine, the aromatic protons, and the α-CH protons.

It is found that, with some exceptions delineated below, the line-widths of the methyl resonances are constant for a particular solvent, independent of the molecular weight of the protein. This indicates that, in general, the proteins behave as random coil structures in these solvents, which confirms the conclusion reached by Tanford and co-workers[1–4] for 6M guanidine hydrochloride.

However, methyl line broadening occurs in dichloroacetic acid for catalase and fibrinogen, in guanidine hydrochloride for insulin, and in urea for insulin and lysozyme. Furthermore, the C 2 histidine resonance is absent in dichloroacetic acid solutions of thyroglobulin, catalase, and fibrinogen; the SCH_3 resonance is absent in myoglobin in trifluoroacetic acid-*d* and occurs as a doublet for trypsin in guanidine hydrochloride and in urea. A general line broadening of resonances indicates association and/or incomplete unfolding of molecules, whereas perturbations of only one particular resonance, as in the cases detailed above, are probably due to intramolecular non-covalent interactions which involve the perturbed group and another unspecified group in the protein.

Introduction

There has been much interest recently in the end product of the unfolding of proteins, viz. the random coil structure in solution with or without disulphide bonds intact.[1–5] This may have been stimulated by earlier work on model compounds for proteins (poly-L-amino acids), which showed that, for polypeptides of a fixed degree of polymerization, the unperturbed end-to-end length in the random coil form is independent of the nature of the side-chain.[6] This has recently been shown to be the case for proteins devoid of disulphide cross links in 6M guanidine hydrochloride and indicates that they do behave as random coils.[1,4,7]

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Theoretical calculations have shown that the dimensions of the random coil would be rather insensitive to the occurrence of "knots" of associated residues produced by non-covalent interactions.[8] However, Tanford and co-workers have also used methods of somewhat greater sensitivity than hydrodynamic methods, viz. optical rotatory dispersion[3] and titration studies,[2] and have found no evidence for the occurrence of non-covalent interactions in 6M guanidine hydrochloride solution.

Nuclear magnetic resonance spectroscopy has not been used to study completely unfolded proteins, except for insulin and its derivatives,[9] but it is a technique which is capable of detecting small changes of conformation in proteins.[10–17] Work with synthetic polymers[18] and poly-γ-benzyl-L-glutamate[19] in the random coil state has shown that the line-width of a resonance is independent of the molecular weight of the polymer and its concentration in the solution. However, with rigid structures there is an increase in line-width with both increase of molecular weight and concentration.[19] Thus for proteins in the random coil form and devoid of non-covalent interactions one might expect to obtain the singlet resonances (viz. those of the C2 protons of histidine, aromatic phenyl protons of phenylalanine, SCH_3 protons of methionine, and CH_3 protons of leucine, valine, and isoleucine) at constant chemical shift and line-width independent of the protein and with an intensity proportional to the number of protons present in the molecule. In this paper we have used these criteria to assess the absence or otherwise of non-covalent interactions in proteins of molecular weight 5700–650000 in a series of denaturing solutions.

Experimental

Insulin (crystalline, bovine pancreas), ribonuclease A (five times crystallized), and bovine thyroglobulin were obtained from Sigma Chemical Co., trypsin (twice crystallized), α-chymotrypsin (three times crystallized), pepsin (twice crystallized), lysozyme (egg white, twice crystallized), and catalase (beef liver) from Worthington Biochemicals, and bovine serum albumin (BSA), bovine fibrinogen, and myoglobin (sperm whale) from Mann Research Laboratories. Poly-L-leucine was obtained from Pilot Chemical Co. (lot No. 6251), L-leucine, L-valine, and L-isoleucine from Nutritional Biochemicals Corp., and guanidine hydrochloride (GuCl) from B.D.H. Solvents used were trifluoroacetic acid-*d* (TFA-*d*) (Merck, spectroscopic grade), D_2O (99·8%, Australian Atomic Energy Commission), and trifluoroacetic acid (TFA), purified as described elsewhere.[20] Dichloroacetic acid (DCA), M. & B. laboratory reagent, was distilled under a reduced pressure

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of nitrogen, and formic acid (FA), analytical reagent, was dried by distillation of a formic acid–sulphuric acid mixture.[21]

Solutions of the amino acids and poly-L-leucine in the acid solvents were 5% (w/v) and all protein solutions were 10% (w/v) except where otherwise stated. Proteins were examined within a few hours of preparation of solutions in TFA-*d*, to minimize chemical reaction,[9] although the n.m.r. spectrum of pepsin was unchanged after 168 hr in TFA-*d*. Solutions of proteins in 6M guanidine hydrochloride and 8M urea in D_2O were titrated with HCl in D_2O or NaOD[12] to the required pH-meter reading.

The n.m.r. spectra were obtained with a Perkin–Elmer 60-MHz R10 spectrometer at 33·4° using an r.f. input setting of 1 mV and sweep rate of 6·4 Hz/sec. Chemical shifts were measured from the internal reference peaks of tetramethylsilane in organic solvents and the sodium salt of 3-trimethylsilyl-1-propanesulphonic acid in D_2O solutions. Relative intensities of resonances were either determined by cutting out the peaks and weighing the paper or by the computer. The base line used was the normal one for all resonances except phenyl and SCH_3, which are sharp spikes superimposed on a large, broad resonance (see Figure 1). In these cases, only the area of the sharp resonance was recorded. A Digital Equipment PDP-8/S computer was used on-line with the spectrometer to increase the signal-to-noise ratio by averaging spectra (40–400 scans were normally collected). The reference peak in the scan was aligned with the reference peak in the averaged spectrum, but in those cases in which this was broadened by interaction with the protein,[22] an external reference was used. The computer was also programmed to estimate chemical shifts, peak widths at half height and intensities of resonances. In addition the computer programme allows for the rejection of unsatisfactory scans and the correction after each scan of baseline drifts and field shifts in the spectrometer.

Results and Discussion

Figure 1 is a typical spectrum of ribonuclease in TFA and the various resonances with which we will be concerned are shown.

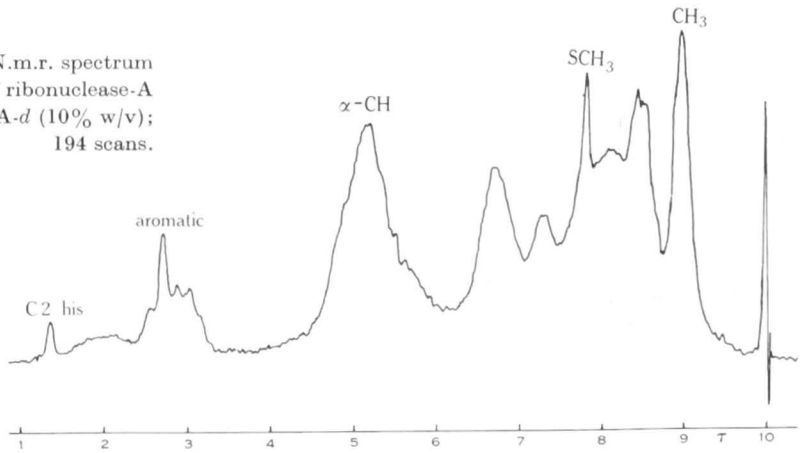

Fig. 1.—N.m.r. spectrum of ribonuclease-A in TFA-*d* (10% w/v); 194 scans.

The assignment of the five resonances of interest are as follows:

C2 his is due to the C2 proton of the imidazole ring of histidine.[9–13,23]

Aromatic resonance is due to the ring protons of phenylalanine, tyrosine, tryptophan, and histidine (C4). The single prominent resonance is due to phenylalanine. Protons attached to nitrogen and recorded elsewhere[9] have been exchanged for deuterons in TFA-*d* and hence are not observed.

[21] Harrap, B. S., and Woods, E. F., *J. Polym. Sci.*, 1961, **49**, 353.

[22] Bradbury, E. M., Crane-Robinson, C., Goldman, H., Rattle, H. W. E., and Stephens, R. M., *J. molec. Biol.*, 1967, **29**, 507.

[23] Bovey, F. A., and Tiers, G. V. D., *J. Am. chem. Soc.*, 1959, **81**, 2870.

α-*CH* resonance includes all α-CH resonances, the α-CH_2 of glycine and the β protons of serine and threonine.[9]

SCH_3 resonance is due to the methyl group of methionine.[9,10,23]

CH_3 resonance is due to the methyl protons of leucine, isoleucine, and valine.[9–11,15,16,23]

We have confirmed the assignment of the α-CH, SCH_3, and CH_3 resonances by experiments with amino acids, some of which are reported below.

TABLE 1

CHEMICAL SHIFTS, LINE-WIDTHS, AND INTENSITIES IN VARIOUS SOLVENTS

Intensities are measured by comparison of the area under the resonance against the area under the α-CH resonance (in TFA-*d* and FA) or the area under the CH_3 peak (in DCA, 6M GuCl, or 8M urea). The figure is reported as a fraction of the theoretical value, which is obtained from the reference numbers following the protein concerned. Abbreviations: dcs, downfield chemical shift; ucs, upfield chemical shift; lb, line broadening; d, doublet

Protein	In TFA-*d*	FA	DCA	6M GuCl[a]	8M Urea[a]
Insulin[24]	dcs α-CH[b]	—	—	dcs C2	
				lb CH_3	lb CH_3
				ucs CH_3	ucs CH_3
Ribonuclease[24]	dcs α-CH	—	—	dcs C2	—
Lysozyme[24]	arom. 0·67	—	—	dcs C2	lb CH_3
	dcs α-CH				
Myoglobin[24]	dcs α-CH	SCH_3 0·70	—	dcs C2	SCH_3 0·75
	no SCH_3			SCH_3 0·72	
Trypsin[24]	dcs α-CH	—	—	dcs C2	
				d SCH_3[c]	d SCH_3[c]
Chymotrypsin[24]	dcs α-CH	—	—	dcs C2	—
Pepsin[25]	dcs α-CH	—	—	—	SCH_3 0·7
BSA[25]	dcs α-CH	—	—	dcs C2	—
Catalase[26]	C2 0·48	—	no C2	dcs C2	C2 0·7
	arom. 0·62		lb CH_3	C2 0·60	
Fibrinogen[27]	dcs α-CH	—	no C2	dcs C2	—
			lb CH_3		
Thyroglobulin[28]	arom. 0·62	—	no C2	dcs C2	—
	dcs α-CH				

[a] pH reading 4–5.

[b] The α-CH resonance is obscured by solvent resonances in all cases except in TFA-*d* and FA and the chemical shift in FA is taken as a reference.

[c] Both peaks were shown to be due to SCH_3 by (i) methylation with methyl iodide after which the doublet disappeared and was replaced by a single resonance at τ 7·02, (ii) mild oxidation with H_2O_2 to produce the sulphoxide which gave a single resonance at τ 7·22.

Occurrence and Chemical Shift

The data for all proteins in the five denaturing solvents are presented in Table 1, in which it is noted when any resonance has an abnormal chemical shift,

[24] Dayhoff, M. O., and Eck, R. V., "Atlas of Protein Sequence and Structure." (National Biomedical Research Foundation: Silver Spring, Md., 1967–68.)

[25] Tristram, G. R., and Smith, R. H., *Adv. Protein Chem.*, 1963, **18**, 227.

[26] Schroeder, W. A., Saha, A., Fenninger, W. D., and Cua, J. T., *Biochim. Biophys. Acta*, 1962, **58**, 611.

[27] Mihalyi, E., Small, P. A., and Cooke, J. P., *Archs Biochem. Biophys.*, 1964, **106**, 229.

[28] Rolland, M., Bismuth, J., Fondarai, J., and Lissitzky, S., *Acta endocr.*, 1966, **53**, 286.

line-width, or intensity. If no entry is made the resonances are quite normal or are obscured by solvent resonances, as occurs in FA for C2 his and in GuCl and urea for the α-CH resonance. Thus the C2 his resonance is not present in thyroglobulin or catalase or fibrinogen in DCA, although it is observed for these proteins in the other solvents, and also for the other proteins in DCA.

The non-occurrence of these resonances may be due to non-covalent interactions which involve the imidazole rings; this would increase the correlation time (decrease the spin–spin relaxation time) of the C2 proton and increase the line-width of the resonance to such an extent that it cannot be observed.[13] Another possibility is that the C2 proton can exchange sufficiently rapidly with the DCA so that the C2 proton would occur in the solvent peak. However, since exchange rates of this proton are normally very slow in acid conditions,[29–31] it is most unlikely that this explanation is correct. The downfield chemical shift of the C2 resonance of about 0·13 p.p.m. which is observed in all proteins in 6M GuCl as compared with the other solvents is thought to be due to specific binding of guanidine to the histidine residues.[32]

The α-CH resonance which is very broad as shown in Figure 1 moves downfield by 0·17 p.p.m. in TFA-*d* over its value in FA for all proteins. This is almost certainly due to additional protonation (charging) of the backbone peptide groups which occurs in the stronger acid TFA-*d* over that in FA. A downfield shift of 0·20–0·35 p.p.m. of the α-CH resonance occurs in model compounds on protonation of the amide group.[33]

The absence of the SCH_3 resonance in myoglobin in TFA-*d* is most likely due to non-covalent interactions between the SCH_3 and an unspecified residue in the protein. Thus, no proteins which we have examined in the native state show an SCH_3 resonance, presumably because of a similar type of interaction, since poly-L-methionine in the helical state of high molecular weight gives a reasonably narrow SCH_3 resonance.[34] A rather more specific interaction is found in the case of trypsin in both GuCl and urea, where a doublet occurs. One resonance is displaced 0·05 p.p.m. upfield from the normal position and is thus due to a perturbed SCH_3 group; possibly the SCH_3 group of Met 166 is magnetically shielded by the adjacent benzene ring of Phe 167. There is a slight upfield shift (about 0·10 p.p.m.) of the methyl resonances of insulin in GuCl and urea which is indicative of incomplete unfolding or association (see below).

Line-Width

The mean line-widths at half height over all proteins and all solvents for which they were visible of the C2 his, phenylalanine, and SCH_3 resonances are 0·09, 0·16, and 0·07 p.p.m. respectively (±0·03 p.p.m.). The line-widths for the α-CH resonances are very large, having mean values of 0·79 p.p.m. in TFA-*d* and 0·65 p.p.m. in FA. Such broad peaks are caused by a number of factors, which are now

[29] Staab, H. A., Wu, M. T., Mannscheck, A., and Schwalbach, G., *Tetrahedron Lett.*, 1964, No. 15, 845.

[30] Olofson, R. A., Thompson, W. R., and Michaelman, J. S., *J. Am. chem. Soc.*, 1964, **86**, 1865.

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considered for the case of CH_3 resonances. Table 2 contains the CH_3 resonances of leucine, isoleucine, and valine which when mixed in equimolar amounts give a complex multiplet resonance with a line-width of 0·19–0·28 p.p.m., depending on the solvent. As shown in Table 2 the breadth of the complex resonance is largely due to the spin–spin splitting of the resonances from each of the amino acids (e.g. leucine) and, to a lesser degree, small chemical shifts between the resonances of the

TABLE 2

LINE-WIDTHS OF METHYL RESONANCES IN PROTEINS[a] AND MODEL COMPOUNDS

Sources of molecular weights are: For pepsin, Dixon, M., and Webb, E. C., "The Enzymes." 2nd Edn. (Longmans: London 1964.) For fibrinogen, Fruton, J. S., and Simmonds, S., "General Biochemistry." 2nd Edn. (John Wiley: London 1958.) For BSA, ref. 25. For the other proteins they are calculated from the amino acid analyses expressed as residues/mole (refs. in Table 1)

Protein or Model Compound	Molecular Weight	$10^2 \times$ Line-Width (p.p.m.) in				
		TFA-*d*	FA	DCA	6M GuCl[b]	8M Urea[b]
Insulin	5700	24	22	29	39	60[e]
Ribonuclease	13700	26	23[c]	38	23	24[d]
Lysozyme	14300	26	21	39	25	65[e]
Myoglobin	17200	24	25	31	23	29
Trypsin	23300	26	24	28	26	27
Chymotrypsin	25800	24	21	39	[f]	23
Pepsin	35000	24	22	30	23	33[g]
BSA	65000	24	23	32	26	24
Catalase	250000	25	24	44	26	28
Fibrinogen	340000	26	26	48	28	38
Thyroglobulin	650000	25	22	37	30	[f]
Mean of proteins		25	23	37	27	35
L-Leu[g]		14	12	14	13	13
poly-L-Leu[a]		17	insol.	insol.	insol.	insol.
L-Leu, L-Val, and L-Ileu[h]		24	25	28	20	19

[a] These resonances always occur as a broad singlet with no observable splitting.

[b] pH reading 4–5.

[c] The line-width in solutions of concentrations 1, 5, 10, and 20% amounts to 0·22, 0·23, 0·23, and 0·24 p.p.m. respectively and is hence independent of concentration.

[d] This line-width amounts to 14 Hz, whereas that obtained at 100 MHz is 17 Hz (0·17 p.p.m.), which indicates only a small contribution to the total line-width due to chemical shift differences.

[e] When pH is reduced to 3·0, the line-width falls to 0·31 p.p.m. for insulin and 0·24 p.p.m. for lysozyme.

[f] Not soluble at the 10% level.

[g] Solution at pH 3·2.

[h] These resonances are split as already described[9,23] and the line-width is therefore very greatly dependent on the degree of spin–spin coupling.

various amino acids. The progression from the small molecule to the macromolecule is seen to cause a small increase in the line-width in most cases and also removal of all the fine structure (splitting) of the resonance. This is shown in the simple case of leucine and poly-L-leucine and also with the amino acid mixture and the CH_3 proton resonance.

It is clear from Table 2 that, except for a few cases detailed below, the line-widths of the methyl resonances in a particular solvent are constant independent of the molecular weight of the protein used. Similar, but less accurate results have been obtained with the C2 his, phenylalanine, and SCH_3 peak widths (see above). It is noted in Table 2 that the methyl line-width is independent of concentration of ribonuclease in FA. These results show that these proteins are in the random coil form, i.e. that rigid structures are absent, since occurrence of the latter would cause increase of line-width with increase of molecular weight and concentration of solute.[19] However, there is evidence from Table 2 of line broadening in DCA for catalase and fibrinogen, in GuCl for insulin and in urea for insulin and lysozyme. This is indicative of association (as shown for insulin in 6M GuCl[35]) and/or incomplete unfolding in these cases.

Since the line-width data of myoglobin (which contains no disulphide bonds) are the same, within experimental error, as those of the other proteins in Table 2, the n.m.r. line-width technique is unable to show the presence of cross links. This contrasts with the sensitivity of hydrodynamic methods, particularly viscometry,[1,36] in detection of changes of shape due to cross links.

Intensities of Resonances

These are summarized in Table 1 for those cases in which the ratio of the intensity of the particular resonance compared with that of the α-CH resonance (in TFA-*d* or FA) or compared with that of the methyl resonance (in DCA, 6M GuCl or 8M urea) deviated from the theoretical ratio (calculated from amino acid analyses) by greater than 25%. Deviations of less than 25%, i.e. values between 0·75 and 1·25, are considered to be within the rather large limits of experimental error of the area measurements and are not recorded. There are a number of cases (particularly with myoglobin) in which the SCH_3 resonance is small or non-existent, also the C2 resonance and to a lesser extent the aromatic resonance. These perturbations are indicative of non-covalent interactions which involve the perturbed group and another unspecified group in the protein, with the possibility of solvent participation.

Since the n.m.r. technique is particularly sensitive in the detection of specific amino acid side-chains, it is very useful in studies (now in progress) of the progressive unfolding of proteins.

Acknowledgments

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Denaturation of Proteins: Single or Multiple Step Process?

We have reported the nuclear magnetic resonance (NMR) spectra of eleven proteins covering a wide range of molecular weights in five denaturing solvents[1]. With some exceptions the proteins (with disulphide bonds intact) appeared to behave in solution as random coils, devoid of non-covalent interactions. We have thus defined by NMR spectroscopy a particular denatured state (the unfolded state) and the next step is to study the denaturation process brought about by the progressive addition of denaturant to the native protein. At 60 MHz we can examine simultaneously six parameters (proton resonances) of the protein[1]. At 220 MHz many more resonances can be observed, because of the much greater resolving power of the spectrometer[2]. Here we direct attention to whether denaturation is a single or multiple step process; a satisfactory answer has often been difficult to obtain in the past[3], but it is a prerequisite of any meaningful thermodynamic study of denaturation.

In general, the denaturation of a protein gives rise to an unfolded conformation, which in some cases undergoes further change, resulting in the formation of an aggregate, precipitate, gel or a refolded non-native conformation (see Fig. 3). When a protein is unfolded, the broad bands in the native spectrum give rise to sharper and better resolved peaks[4–7]. The fraction of unfolding F is defined by the equation $F=(h-h_N)/(h_U-h_N)$, where h_N, h and h_U are the heights of the particular NMR resonance for the native protein, for the protein at the measured concentration of denaturant and for the completely unfolded protein respectively. For a single step process the spectrum in the transition region is a linear combination of the spectra of the native and unfolded molecules. If intermediates are present during unfolding, they may be recognized by (*a*) the appearance of additional resonances in the transition region and/or (*b*) the non-equivalence of F for various peaks at a particular concentration of denaturant. Subsequent conformational changes after the initial unfolding are readily detected, because in all the cases described, they would lead to a decrease in F.

The experimental technique consists of the measurement of the NMR spectrum at 60 MHz[1] of a native protein in solution, followed by successive additions of denaturant and at least 3 h accumulation of the spectrum after each addition. The assignment of peaks has been described[1] (see also Fig. 2) except for the peptide and arginine NH peaks[8].

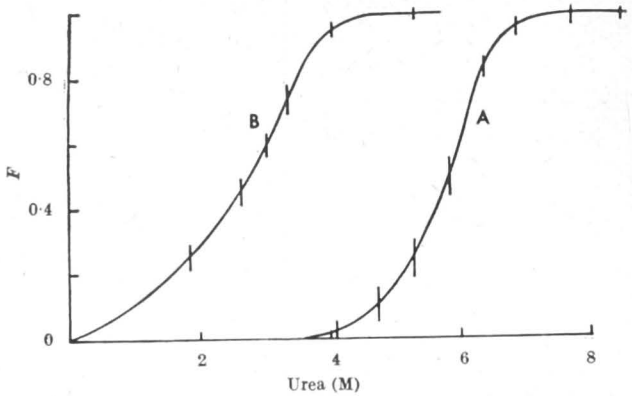

Fig. 1. Denaturation of ribonuclease in aqueous urea at (*A*) *p*H 4·7, (*B*) *p*H 2·8. The vertical lines represent standard deviations from the mean value of *F* obtained as an average from six peaks.

The denaturation of ribonuclease at *p*H 4·7 in aqueous urea occurs in a single step, because the values of *F* obtained from all six peaks (C–2 histidine, peptide NH, phenyl protons of phenylalanine, arginine NH, SCH_3 of methionine and methyl protons of valine, isoleucine and leucine) are constant at a particular urea concentration (Fig. 1*A*). The graph of *F* against molarity of urea is a

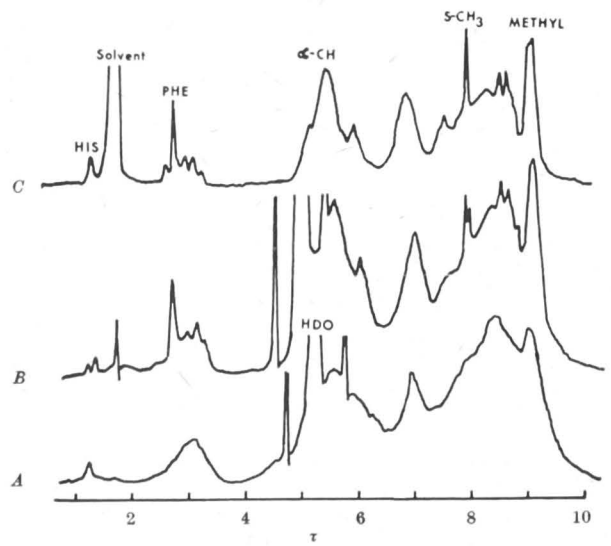

Fig. 2. NMR spectra of ribonuclease (10–15 per cent) in (*A*) D_2O, (*B*) D_2O containing 10 per cent d_2-formic acid, (*C*) D_2O containing 80 per cent d_2-formic acid (v/v). Note the occurrence of an upfield histidine resonance which corresponds to the unfolded state of the protein.

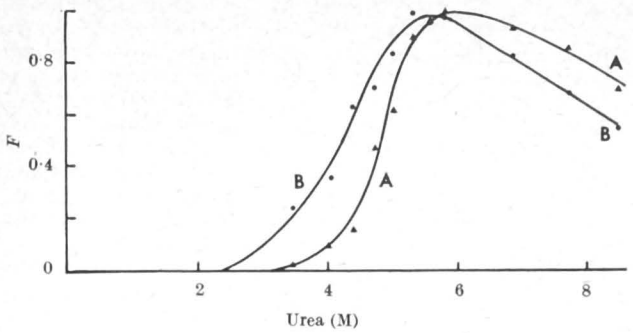

Fig. 3. Denaturation of lysozyme by urea at *p*H 2·8. (*A*) represents average *F* values for the S–CH_3 and methyl peaks, (*B*) *F* values for the arginine NH peak which is also followed with less accuracy by the height of the upfield histidine peak produced on denaturation (see Fig. 2).

symmetrical sigmoidal curve at *p*H 4·7, but is markedly asymmetric at *p*H 2·8 (Fig. 1). Such skewness in transition curves has been attributed to the existence of intermediates[9], but there is no evidence in either case for the occurrence of additional peaks during the transition.

Additional peaks are observed on the denaturation of ribonuclease in D_2O by the addition of d_2-formic acid. For native ribonuclease in D_2O (Fig. 2*A*) no methionine peak is observed. In the transition region (Fig. 2*B*) two resonances arising from the S–CH_3 protons of methionine residues are present. The upfield peak, with an abnormal chemical shift, corresponds to an intermediate, partially unfolded conformation. The height of the upfield resonance decreases to zero as the unfolding approaches completion (Fig. 2*C*). Similar intermediates, characterized by an upfield methionine peak, were observed in the denaturation of ribonuclease by hydrochloric acid and by potassium thiocyanate. We therefore conclude that the unfolding of ribonuclease by these denaturants is not a single-step process.

The denaturation of hen egg-white lysozyme by urea in water at *p*H 2·8 demonstrates yet another type of behaviour (Fig. 3). The value of *F* at each urea concentration between 2·5 and 6 M is greater for the arginine and histidine peaks than for the methionine and upfield methyl peaks. This shows that those regions of the lysozyme molecule which contain an abundance of histidine and arginine residues, unfold at lower urea concentrations than other portions of the molecule rich in aliphatic side-chains. These latter portions may be identified with the hydrophobic core of lysozyme[10]. Furthermore, the arginine and histidine residues are all on the surface of the lysozyme molecule[10]. It therefore appears that the unfolding of lysozyme by urea proceeds in at least two

steps, with the outer portion of the molecule unfolding before the hydrophobic core. The decrease in F above 6 M is probably caused by aggregation, because a gel formed from the 8·5 M solution after a few hours.

Thus, by observation of six parameters (resonances) simultaneously, the denaturation of ribonuclease in urea at pH 4·7 (and perhaps also at pH 2·8) appears to occur in a single step, whereas in KCNS or in acids (formic acid and HCl) multiple steps are observed. With lysozyme at pH 2·8 the exterior of the molecule unfolds in urea before the hydrophobic core. It is clear that diverse results are obtained for one protein (ribonuclease) in different denaturants and for different proteins (ribonuclease and lysozyme) in the same denaturant. The NMR technique can, however, detect the occurrence of intermediate states; it is clearly the most powerful technique currently available for structural studies of proteins in solution.

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